PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C07H 21/04, C07K 14/435 — A1

(11) International Publication Number: WO 96/33206

(43) International Publication Date: 24 October 1996 (24.10.96

(21) International Application Number: PCT/US96/05262

(22) International Filing Date: 17 April 1996 (17.04.96)

(30) Priority Data:

08/423,561	17 April 1995 (17.04.95)	US
08/599,556	15 February 1996 (15.02.96)	US
08/624,123	29 March 1996 (29.03.96)	US

(71) Applicant: UNIVERSITY OF UTAH RESEARCH FOUNDATION [US/US]; Suite 170, 421 Wakara Way, Salt Lake City, UT 84108 (US).

(72) Inventors: SHON, Ki-Joon; University of Utah, Biology Dept., 201 South Biology Building, Salt Lake City, UT 84112 (US). GRILLEY, Michelle, M.; University of Utah, Biology Dept., 201 South Biology Building, Salt Lake City, UT 84112 (US). OLIVERA, Baldomero, M.; 1370 Bryan Avenue, Salt Lake City, UT 84108 (US). YOSHIKAMI, Doju; 1074 East 600 South, Salt Lake City, UT 84111 (US). MARSH, Maren; 845 East Garfield Avenue, Salt Lake City, UT 84105 (US). CRUZ, Lourdes, J.; 31 M Street #403, Salt Lake City, UT 84114 (US). HILLYARD, David, R.; 3685 Juno Circle, Salt Lake City, UT 84124 (US).

(74) Agents: IHNEN, Jeffrey, L. et al.; Venable, Baetjer, Howard & Civiletti, L.L.P., 1201 New York Avenue, N.W., # 1000, Washington, DC 20005 (US).

(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: CONOTOXIN PEPTIDES

SEQ ID NO: 12

⇓

ZRLCCGFOKSCRSRQCKOHRCC-NH_2

SEQ ID NO: 2

(57) Abstract

The present invention is directed to conotoxin peptides, specifically δ-conotoxin PVIA and μ-conotoxin PIIIA. δ-conotoxin PVIA is found in the Eastern Pacific fish hunting species of cone snails *Conus purpurascens*. It consists of 29 amino acid residues of the sequence: Glu-Ala-Cys-Tyr-Ala-Xaa_1-Gly-Thr-Phe-Cys-Gly-Ile-Lys-Xaa_2-Gly-Leu-Cys-Cys-Ser-Glu-Phe-Cys-Leu-Pro-Gly-Val-Cys-Pro-Gly (SEQ ID NO:1) where Xaa_1 or Xaa_2 is Pro or 4-trans-hydroxyproline. The C-terminus may be free or amidated. δ-conotoxin PVIA is vertebrate specific and targets voltage-sensitive Na^+ channels. μ-conotoxin PIIIA is also found in *Conus purpurascens*. It consists of 22 amino acid residues of the sequence: Xaa_1-Arg-Leu-Cys-Cys-Gly-Phe-Xaa_2-Lys-Ser-Cys-Arg-Ser-Arg-Gln-Cys-Lys-Xaa_2-His-Arg-Cys-Cys (SEQ ID NO:2) where Xaa_1 represents pyroglutamate or glutamine and Xaa_2 represents 4-trans-hydroxyproline or proline. This latter peptide targets sodium channels.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia
AT	Austria
AU	Australia
BB	Barbados
BE	Belgium
BF	Burkina Faso
BG	Bulgaria
BJ	Benin
BR	Brazil
BY	Belarus
CA	Canada
CF	Central African Republic
CG	Congo
CH	Switzerland
CI	Côte d'Ivoire
CM	Cameroon
CN	China
CS	Czechoslovakia
CZ	Czech Republic
DE	Germany
DK	Denmark
EE	Estonia
ES	Spain
FI	Finland
FR	France
GA	Gabon
GB	United Kingdom
GE	Georgia
GN	Guinea
GR	Greece
HU	Hungary
IE	Ireland
IT	Italy
JP	Japan
KE	Kenya
KG	Kyrgyzstan
KP	Democratic People's Republic of Korea
KR	Republic of Korea
KZ	Kazakhstan
LI	Liechtenstein
LK	Sri Lanka
LR	Liberia
LT	Lithuania
LU	Luxembourg
LV	Latvia
MC	Monaco
MD	Republic of Moldova
MG	Madagascar
ML	Mali
MN	Mongolia
MR	Mauritania
MW	Malawi
MX	Mexico
NE	Niger
NL	Netherlands
NO	Norway
NZ	New Zealand
PL	Poland
PT	Portugal
RO	Romania
RU	Russian Federation
SD	Sudan
SE	Sweden
SG	Singapore
SI	Slovenia
SK	Slovakia
SN	Senegal
SZ	Swaziland
TD	Chad
TG	Togo
TJ	Tajikistan
TT	Trinidad and Tobago
UA	Ukraine
UG	Uganda
US	United States of America
UZ	Uzbekistan
VN	Viet Nam

TITLE OF THE INVENTION

CONOTOXIN PEPTIDES

This invention was made with Government support under Grant No. PO1 GM 48677 awarded by the National Institutes of Health, Bethesda, Maryland. The United States Government has certain rights in the invention.

BACKGROUND OF THE INVENTION

This invention relates to relatively short peptides, and more particularly to peptides between about 22 and about 35 residues in length, which are naturally available in minute amounts in the venom of the cone snails or analogous to the naturally available peptides, and which include three cyclizing disulfide linkages.

The publications and other materials used herein to illuminate the background of the invention, and in particular, cases to provide additional details respecting the practice, are incorporated by reference, and for convenience are referenced in the following text by author and date and are listed alphabetically by author in the appended bibliography.

Mollusks of the genus *Conus* produce a highly toxic venom which enables them to carry out their unique predatory lifestyle. Prey are immobilized by the venom which is injected by means of a highly specialized venom apparatus, a disposable hollow tooth which functions both in the manner of a harpoon and a hypodermic needle.

Few interactions between organisms are more striking than those between a venomous animal and its envenomated victim. Venom may be used as a primary weapon to capture prey or as a defense mechanism. These venoms disrupt essential organ systems in the envenomated animal, and many of these venoms contain molecules directed to receptors and ion channels of neuromuscular systems.

The predatory cone snails (*Conus*) have developed a unique biological strategy. Their venom contains relatively small peptides that are targeted to various neuromuscular receptors and may be equivalent in their pharmacological diversity to the alkaloids of plants or secondary metabolites of microorganisms. Many of these peptides are among the smallest nucleic acid-encoded translation products having defined conformations, and as such, they are somewhat unusual. Peptides in this size range normally equilibrate among many conformations. Proteins having a fixed conformation are generally much larger.

The cone snails that produce these toxic peptides, which are generally referred to as conotoxins or conotoxin peptides, are a large genus of venomous gastropods comprising approximately 500 species. All cone snail species are predators that inject venom to capture prey, and the spectrum of animals that the genus as a whole can envenomate is broad. A wide variety of hunting strategies are used; however, every *Conus* species uses fundamentally the same basic pattern of envenomation.

The major paralytic peptides in these fish-hunting cone venoms were the first to be identified and characterized. In *C. geographus* venom, three classes of disulfide-rich peptides were found: the α-conotoxin peptides (which target and block the nicotinic acetylcholine receptors); the μ-conotoxin peptides (which target and block the skeletal muscle Na^+ channels); and the ω-conotoxin peptides (which target and block the presynaptic neuronal Ca^{2+} channels). However, there are multiple homologs in each toxin class; for example, there are at least five different ω-conotoxin peptides present in *C. geographus* venom alone. Considerable variation in sequence is evident, and when different ω-conotoxin peptide sequences were first compared, only the cysteine residues that are involved in disulfide bonding and one glycine residue were found to be invariant. Another class of conotoxins found in *C. geographus* venom is that referred to as conantokins, which cause sleep in young mice and hyperactivity in older mice and are targeted to the NMDA receptor. Each cone venom appears to have its own distinctive group, or signature, of different conotoxin sequences.

Many of these peptides have now become fairly standard research tools in neuroscience and can be used as chemical probes for receptors and ion channels (Myers et al., 1993). μ-Conotoxin peptides, because of their ability to preferentially block muscle but not axonal Na^+ channels, are convenient tools for immobilizing skeletal muscle without affecting axonal or synaptic events. ω-Conotoxin peptides have become standard pharmacological reagents for investigating voltage-sensitive Ca^{2+} channels and are used to block presynaptic termini and neurotransmitter release. Several conotoxin peptides have also found utility in screening newly isolated conotoxin peptides or analogs for medical purposes (Miljanich et al., 1993).

Many potent toxins target voltage-gated sodium channels; these have been indispensable for investigating the structure and function of these ion channels which play a key role in excitable tissues. The demonstration that tetrodotoxin specifically inhibited voltage-gated sodium currents without effect on potassium currents provided crucial experimental support for the Hodgkin-

Huxley formulation of action potential generation (Narahashi et al., 1964). A variety of ligands for the Na^+ channel has been discovered since, and their sites of binding and modes of activity have been investigated (Catterall, 1992).

Site I is the classical binding site for channel blockers, notably the guanidinium toxins, saxitoxin (STX) and tetrodotoxin (TTX); this site is generally postulated to be at the extracellular end of the channel pore. Only one family of polypeptide toxins, the μ-conotoxins, has been shown to act at this site and functionally affect voltage-gated sodium currents. These were originally isolated from the venom of the marine snail *Conus geographus* (Stone and Gray, 1982; Sato et al., 1983; Cruz et al., 1985; and Olivera et al., 1985).

Other families of *Conus* peptides (notably the ω-conotoxins which target calcium channels and the α-conotoxins which target nicotinic acetylcholine receptors) have been found in the venoms of many *Conus* species examined. They show extreme variability among homologous peptides from different *Conus* species, and interspecific comparison of different members within a given family of *Conus* peptides has provided insightful structure-function information. In particular, the wide diversity among natural toxins in these families has been instrumental in identifying new classes of receptors (Olivera et al., 1990; Olivera et al., 1994). By contrast, because the μ-conotoxins have so far been described only from the venom of *C. geographus*, most structure-function information for this peptide family has come from experiments with synthetic analogs.

One aspect of the invention is directed to δ-conotoxin PVIA having the formula Glu-Ala-Cys-Tyr-Ala-Xaa_1-Gly-Thr-Phe-Cys-Gly-Ile-Lys-Xaa_2-Gly-Leu-Cys-Cys-Ser-Glu-Phe-Cys-Leu-Pro-Gly-Val-Cys-Pro-Gly (Xaa_1 or Xaa_2 is Pro or *4-trans*-hydroxyproline) (SEQ ID NO:1). The C-terminus may be free or amidated. This conotoxin is vertebrate-specific and targets voltage-sensitive Na channels.

A second aspect of the present invention describes a new member of the μ-conotoxin peptide family - μ-conotoxin PIIIA from *Conus purpurascens*, an Eastern Pacific fish-hunting species. As expected, the new μ-conotoxin shows considerable sequence divergence from the μ-conotoxins of *Conus geographus*. In addition to a comprehensive biochemical characterization of the peptide, there is provided electrophysiological and binding data which demonstrate that μ-conotoxin PIIIA is a powerful pharmacological tool for distinguishing among different tetrodotoxin-sensitive Na^+ channel subtypes. The tetrodotoxin-sensitive sodium channels can now be resolved into three

categories: 1) sensitive to μ-PIIIA and μ-conotoxin GIIIA; 2) sensitive to μ-PIIIA but not to μ-GIIIA; and 3) sensitive to neither μ-conotoxin (examples are skeletal muscle, rat brain Type II and motor axon subtypes, respectively). In rat brain, binding competition studies between the two μ-conotoxins and [^{3}H]saxitoxin suggest at least three pharmacologically distinguishable binding sites. Thus, μ-conotoxin PIIIA is a key tool for distinguishing among different sodium channel subtypes.

SUMMARY OF THE INVENTION

One aspect of the present invention is directed to conotoxin peptides having 25-35 amino acids, six cysteines which form three disulfide bonds between the first and fourth, second and fifth, and third and sixth cysteines, respectively. The invention is directed to δ-conotoxin PVIA having the formula Glu-Ala-Cys-Tyr-Ala-Xaa$_1$-Gly-Thr-Phe-Cys-Gly-Ile-Lys-Xaa$_2$-Gly-Leu-Cys-Cys-Ser-Glu-Phe-Cys-Leu-Pro-Gly-Val-Cys-Pro-Gly (Xaa$_1$ or Xaa$_2$ is Pro or *4-trans*-hydroxyproline) (SEQ ID NO:1). The C-terminus may be free or amidated. This conotoxin is vertebrate-specific and targets voltage-sensitive Na channels.

A second aspect of the present invention is directed to conotoxin peptides having 22 amino acids, six cysteines which form three disulfide bonds between the first and fourth, second and fifth, and third and sixth cysteines, respectively. The invention is directed to μ-conotoxin PIIIA having the formula Xaa$_1$-Arg-Leu-Cys-Cys-Gly-Phe-Xaa$_2$-Lys-Ser-Cys-Arg-Ser-Arg-Gln-Cys-Lys-Xaa$_2$-His-Arg-Cys-Cys (SEQ ID NO:2) where Xaa$_1$ represents pyroglutamate or glutamine and Xaa$_2$ represents 4-trans-hydroxyproline or proline. This peptide targets sodium channels.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the nucleic acid sequence and predicted amino acid sequence of a cDNA clone encoding the lockjaw peptide precursor and a comparison of the lockjaw peptide to two other related *Conus* peptide precursor sequences. The sequences labeled 1, 2 and 3 in this figure are precursors of ω-conotoxin GVIA, δ-conotoxin TxVIa and δ-conotoxin PVIA respectively.

Figure 2A shows the nucleic acid sequence derived by analyzing cDNA clones from a *Conus purpurascens* venom duct library. The sequence encoding the inferred C-terminal end of the open

reading frame is shown; mature conotoxins are always encoded at the C-terminus of the precursor sequence. The pattern of Cys residues suggested that the encoded C-terminal peptide might be a μ-conotoxin. The arrow indicates the predicted site of proteolytic cleavage to generate the mature toxin. A -Lys-Arg- sequence is the most common motif for proteolytic cleavage of conotoxin precursors.

Figure 2B shows the predicted sequence of the post-translationally processed peptide. The amino acid sequence shown in Figure 2A would be predicted to be post-translationally processed at the four indicated sites as follows: proline would be hydroxylated to 4-trans-hydroxyproline (sites 2 and 3); the C-terminal -Cys-Gly-Arg- sequence would be processed by an exopeptidase and amidation enzymes to a -Cys-NH_2 moiety (site 4); and after proteolysis the encoded glutamine residue would be converted to pyroglutamate (site 1). The post-translational processing events would yield the indicated bold sequence (Z = pyroglutamate, O = 4 trans-hydroxyproline).

Figure 3A is a sketch of electrophysiological recording chamber for testing toxin on frog cutaneus pectoris muscle's response to direct electrical stimulation. A rectangular Sylgard trough was partitioned into four compartments (**A** through **D**) by three Mylar sheets (**1-3**). Mylar sheets were inserted into slots in the wall of the trough after the muscle had been pinned to the floor of the trough. The cutaneus end of the muscle was in **A** and the episternum (cartilage) was in **D**. Stimulating electrodes were in **A** and **B** (i.e., stimulation was across partition **1**). A ground electrode was in **B**. Recording electrodes were in **C** and **D** (with electrode in **D** leading to the "+" input of the preamp). Compartment **D** served as the test chamber -- only it was exposed to toxin.

Figure 3B shows that PIIIA at a concentration of 1 μM blocks directly-evoked action potentials in frog muscle. Superimposed traces of responses before, during and after exposure to toxin are shown. The stimulus was applied at t=0. Curve 1 is for a control sample response. Curve 2 shows a response after exposure to toxin for 23 minutes and just before the toxin was washed out. Curve 3 shows the response 20 minutes after toxin washout. Curve 4 shows the response after >4.5 hours of washing. Toxin was placed only in one compartment (D in Figure 3A), and it contained the portion of the muscle which produced the negative phase of the response in the control trace.

Figure 3C shows the time course of block of directly-evoked action potentials. Maximum amplitudes of the positive phase (open circles) and negative phase (closed circles) of the response

are plotted as a function of time. Solution in compartment D was replaced with 1 μM PIIIA at time 0 (downward arrow), and the toxin was washed out 23 minutes later (upward arrow).

Figure 4 shows the results of binding competition experiments with [^{3}H]saxitoxin (SXT). Specific binding was determined by subtracting nonspecific binding of [^{3}H]saxitoxin from total binding; the nonspecific binding was measured by using 12 μM tetrodotoxin (TTX) to displace [^{3}H]saxitoxin binding. Open circles, μ-PIIIA displacement for eel electroplax sites; squares, μ-PIIIA displacement for rat brain sites; triangles, μ-GIIIA displacement for rat brain sites

Figures 5A, 5B and 5C show that μ-PIIIA blocks rat type II Na^+ channel expressed in *Xenopus* oocytes. Figure 5A shows whole cell current recorded from an oocyte expressing rat type II Na^+ channels. Voltage steps ranging from -80 mV to +60 mV, in 10 mV increments, were generated from a holding potential of -100 mV. Figure 5B shows results (profound block of the currents) when 2 mM PIIIA was added to the bath solution. Figure 5C shows the results following a wash with Normal Frog Ringers solution.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

The present invention is directed to conotoxin peptides having 22-35 amino acids, six cysteines which form three disulfide bonds between the first and fourth, second and fifth, and third and sixth cysteines, respectively, and to the precursors of these peptides. One aspect of the invention is directed to δ-conotoxin PVIA having the formula Glu-Ala-Cys-Tyr-Ala-Xaa_1-Gly-Thr-Phe-Cys-Gly-Ile-Lys-Xaa_2-Gly-Leu-Cys-Cys-Ser-Glu-Phe-Cys-Leu-Pro-Gly-Val-Cys-Pro-Gly (Xaa_1 or Xaa_2 is Pro or *4-trans*-hydroxyproline) (SEQ ID NO:1). The C-terminus may be free or amidated. This conotoxin is vertebrate-specific and targets voltage-sensitive Na channels and is useful as an active agent for muscle contraction in instances where lack of muscle contraction is problematic, such as for treating urinary or fecal incontinence. This conotoxin is also useful for tagging tumors since it is able, as are other conotoxins, to detect antibodies which form against tumors. Since conotoxins bind to cell surface receptors, ion channels, they are capable of inhibiting tumor growth and are useful as anti-neoplastic agents.

Despite the close relationship of the δ-conotoxins to the ω-conotoxins, it is clear that they have different physiological targets. The ω-conotoxins inhibit voltage-gated Ca^{2+} channels, distinguishing various subtypes. In contrast, the δ-conotoxins are without effect on Ca^{2+} channels;

the results shown below demonstrate that that they do not compete for binding with ω-conotoxin GVIA and do not induce the shaking syndrome in mice characteristic of the ω-conotoxins.

Biologically active δ-conotoxin GmVIA has been chemically synthesized, demonstrating that the biological activity is not due to contaminants. A different family of *Conus* peptides, the μ-conotoxins, is known which also affects Na^+ channels. However, these have a different disulfide framework, are channel blockers specific for the muscle subtype, and, like the ω-conotoxins, are highly basic molecules. Given the very different chemical character of δ-conotoxins, it is likely that their site of action on the Na^+ channel is quite distinct.

Biologically active δ-conotoxin PVIA has been chemically synthesized, demonstrating that the biological activity is not due to contaminants. δ-Conotoxin PVIA (sometimes called herein the lockjaw peptide), present in the venom of the fish hunting snail *C. purpurascens*, is the first biochemically characterized toxin shown to underlie such symptoms, as well as to increase excitability at the vertebrate neuromuscular junction. When the peptide was injected into fish intraperitoneally or intramuscularly, a characteristic rapid and very jerky swimming behavior was followed by rigid paralysis, the lockjaw syndrome, and death. Excitotoxin activities were also induced upon intracranial injection of the peptide into mice.

The data presented below strongly indicate that the lockjaw peptide is a vertebrate-targeted δ-conotoxin. The *C. purpurascens* lockjaw peptide was inactive in the molluscan test system at doses 100-fold higher than required to potently affect both fish and mice. In contrast, δ-conotoxin TxVIA which potently potentiates molluscan Na channels showed no biological activity in any assays involving vertebrate systems.

Nevertheless, δ-conotoxin TxVIA and the lockjaw peptide competed for the same binding site in rat brain membranes. δ-Conotoxin TxVIA binds specifically and with high affinity to voltage-sensitive sodium channels in the mammalian central nervous system, even though it has no inhibitory effect (Fainzilber et al., 1994). Taken together, the primary structure of the lockjaw peptide, the predicted amino acid sequence of the precursor, the electrophysiological results using the frog neuromuscular junction, the binding data, and the *in vivo* symptoms induced by the peptide are consistent with the conclusion that the lockjaw peptide is a vertebrate-targeted δ-conotoxin. In contrast to previously characterized δ-conotoxins (TxVIA and GmVIA) which had no effects on cloned rat brain Na channels, δ-conotoxin PVIA had clear effects on this mammalian channel. Under depolarizing conditions,

the toxin inhibited channel inactivation. A persistent conductance was observed in the presence of this peptide. That is, when voltage is brought from a resting potential to 0 mV and held there for 18 mseconds, virtually no conductance remains in the control, whereas a residual Na conductance remains in the presence of δ-conotoxin PVIA.

The cloning data described below demonstrate that the toxin precursor must have an amidated C-terminus. The amidated lockjaw peptide is designated as δ-conotoxin PVIA and the form of the peptide with the free carboxyl terminus as [deamido]-δ-conotoxin PVIA. It should be noted that another fish-hunting *Conus* species, *Conus striatus*, has a toxin which apparently acts by the same physiological mechanism. However, the purified toxin from *C. striatus* (striatotoxin) has been reported to have a much higher molecular mass (≈25 kDa) (Kobayashi et al., 1982) which would appear to make it distinct from all of the δ-conotoxins characterized so far.

In a survey of a dozen *Conus* venoms, a broad spectrum of mammalian excitotoxins was found to be present. Previously characterized conotoxins (α-, μ- and ω-) all cause a decrease in electrical excitability or an inhibition of neurotransmission (Myers et al., 1993; Olivera et al., 1990). The comprehensive biochemical characterization and successful chemical synthesis of δ-conotoxin PVIA is the first description of a vertebrate-targeted excitotoxin from *Conus* venoms.

A second aspect of the invention is directed to μ-conotoxin PIIIA having the formula Xaa_1-Arg-Leu-Cys-Cys-Gly-Phe-Xaa_2-Lys-Ser-Cys-Arg-Ser-Arg-Gln-Cys-Lys-Xaa_2-His-Arg-Cys-Cys (SEQ ID NO:2) where Xaa_1 represents pyroglutamate or glutamine and Xaa_2 represents 4-trans-hydroxyproline or proline and is also directed to precursors of these peptides and nucleic acids encoding these peptides. This peptide is a sodium channel blocker and is useful as an active agent for muscle contraction in instances where lack of muscle contraction is problematic, such as for treating urinary or fecal incontinence. It is also useful as an active agent for anti-seizures, e.g., an anti-epileptic. Further, this conotoxin may also be useful for tagging tumors since they are able, as are other conotoxins, to detect antibodies which form against tumors. Since conotoxins

bind to cell surface receptors, ion channels, they are capable of inhibiting tumor growth and are useful as anti-neoplastic agents.

Chemical Synthesis of Conotoxins

These peptides are sufficiently small to be chemically synthesized. General chemical syntheses for preparing the foregoing conotoxin peptides are described hereinafter, along with specific chemical syntheses of several conotoxin peptides and indications of biological activities of these synthetic products. Various ones of these conotoxin peptides can also be obtained by isolation and purification from specific *Conus* species using the technique described in U.S. Patent No. 4,447,356 (Olivera et al., 1984), the disclosure of which is incorporated herein by reference.

Although the conotoxin peptides of the present invention can be obtained by purification from cone snails, because the amounts of conotoxin peptides obtainable from individual snails are very small, the desired substantially pure conotoxin peptides are best practically obtained in commercially valuable amounts by chemical synthesis. For example, the yield from a single cone snail may be about 10 micrograms or less of conotoxin peptide. By "substantially pure" is meant that the peptide is present in the substantial absence of other biological molecules of the same type; it is preferably present in an amount of at least about 85% by weight and preferably at least about 95% of such biological molecules of the same type which are present (i.e., water, buffers and innocuous small molecules may be present). Chemical synthesis of biologically active conotoxin peptides depends of course upon correct determination of the amino acid sequence.

The conotoxin peptides can also be produced by recombinant DNA techniques well known in the art. Such techniques are described by Sambrook et al. (1979). The peptides produced in this manner are isolated, reduced if necessary, and oxidized to form the correct disulfide bonds.

One method of forming disulfide bonds in the conotoxin peptides of the present invention is the air oxidation of the linear peptides for prolonged periods under cold room temperatures. This procedure results in the creation of a substantial amount of the bioactive, disulfide-linked peptides. The oxidized peptides are fractionated using reverse-phase high performance liquid chromatography (HPLC) or the like, to separate peptides having different linked configurations. Thereafter, either by comparing these fractions with the elution of the native material or by using a simple assay, the particular fraction having the correct linkage for maximum biological potency is easily determined. It is also found that the linear peptide, or the oxidized product having more than

one fraction, can sometimes be used for *in vivo* administration because the cross-linking and/or rearrangement which occurs *in vivo* has been found to create the biologically potent conotoxin molecule. However, because of the dilution resulting from the presence of other fractions of less biopotency, a somewhat higher dosage may be required.

A second method of forming the disulfide bonds in the conotoxin peptides of the present invention involves the use of acetamidomethyl (Acm) as protection agent on the second and fifth cysteines during the synthesis of the conotoxin peptides. The use of Acm on these two residues is based on the analogy with disulfide bridges in other conotoxin peptides. The peptide with the Acm protected cysteines is air-oxidized overnight at room temperature. The bicyclic peptides are separated by high performance liquid chromatography (HPLC) and the desired isomer isolated. The final disulfide bridge is carried out by iodination. The undesired isomers are efficiently recycled by reduction to linear peptide. The desired isomer is determined by a partial reduction analysis (Gray, 1993). In this analysis, a sample of a bicyclic precursor is treated with tris-[2-carboxyethyl]-phosphine to give linear peptide and a singly-bridged intermediate. The latter peptide is reacted with iodoacetamide, and the location of alkylated cysteine residues is established by sequence analysis. In this analysis, it was determined that the correct linkages were between the first and fourth, second and fifth, and third and sixth cysteines for PVIA, for example.

The peptides are synthesized by a suitable method, such as by exclusively solid-phase techniques, by partial solid-phase techniques, by fragment condensation or by classical solution couplings. The employment of recently developed recombinant DNA techniques may be used to prepare these peptides, particularly the longer ones containing only natural amino acid residues which do not require post-translational processing steps.

In conventional solution phase peptide synthesis, the peptide chain can be prepared by a series of coupling reactions in which the constituent amino acids are added to the growing peptide chain in the desired sequence. The use of various N-protecting groups, e.g., dicyclohexylcarbodiimide or carbonyldimidazole, various active esters, e.g., esters of N-hydroxyphthalimide or N-hydroxy-succinimide, and the various cleavage reagents, to carry out reaction in solution, with subsequent isolation and purification of intermediates, is well known classical peptide methodology. Classical solution synthesis is described in detail in the treatise "Methoden der Organischen Chemie (Houben-Weyl): Synthese von Peptiden," (1974). Techniques of exclusively solid-phase synthesis are set forth in the textbook, "Solid-Phase Peptide Synthesis,"

(Stewart and Young, 1969), and are exemplified by the disclosure of U.S. Patent 4,105,603 (Vale et al., 1978). The fragment condensation method of synthesis is exemplified in U.S. Patent 3,972,859 (1976). Other available syntheses are exemplified by U.S. Patents No. 3,842,067 (1974) and 3,862,925 (1975).

Common to such chemical syntheses is the protection of the labile side chain groups of the various amino acid moieties with suitable protecting groups which will prevent a chemical reaction from occurring at that site until the group is ultimately removed. Usually also common is the protection of an α-amino group on an amino acid or a fragment while that entity reacts at the carboxyl group, followed by the selective removal of the α-amino protecting group to allow subsequent reaction to take place at that location. Accordingly, it is common that, as a step in such a synthesis, an intermediate compound is produced which includes each of the amino acid residues located in its desired sequence in the peptide chain with appropriate side-chain protecting groups linked to various ones of the residues having labile side chains.

As far as the selection of a side chain amino protecting group is concerned, generally one is chosen which is not removed during deprotection of the α-amino groups during the synthesis. However, for some amino acids, e.g., His, protection is not generally necessary. In selecting a particular side chain protecting group to be used in the synthesis of the peptides, the following general rules are followed: (a) the protecting group preferably retains its protecting properties and is not split off under coupling conditions, (b) the protecting group should be stable under the reaction conditions selected for removing the α-amino protecting group at each step of the synthesis, and (c) the side chin protecting group must be removable, upon the completion of the synthesis containing the desired amino acid sequence, under reaction conditions that will not undesirably alter the peptide chain.

It should be possible to prepare many, or even all, of these peptides using recombinant DNA technology. However, when peptides are not so prepared, they are preferably prepared using the Merrifield solid-phase synthesis, although other equivalent chemical syntheses known in the art can also be used as previously mentioned. Solid-phase synthesis is commenced from the C-terminus of the peptide by coupling a protected α-amino acid to a suitable resin. Such a starting material can be prepared by attaching an α-amino-protected amino acid by an ester linkage to a chloromethylated resin or a hydroxymethyl resin, or by an amide bond to a benzhydrylamine (BHA) resin or paramethylbenzhydrylamine (MBHA) resin. Preparation of the hydroxymethyl

resin is described by Bodansky et al., (1966). Chloromethylated resins are commercially available from Bio Rad Laboratories (Richmond, CA) and from Lab. Systems, Inc. The preparation of such a resin is described by Stewart et al. (1969). BHA and MBHA resin supports are commercially available, and are generally used when the desired polypeptide being synthesized has an unsubstituted amide at the C-terminus. Thus, solid resin supports may be any of those known in the art, such as one having the formulae -O-CH_2-resin support, -NH BHA resin support, or -NH-MBHA resin support. When the unsubstituted amide is desired, use of a BHA or MBHA resin is preferred, because cleavage directly gives the amide. In case the N-methyl amide is desired, it can be generated from an N-methyl BHA resin. Should other substituted amides be desired, the teaching of U.S. Patent No. 4,569,967 (Kornreich et al., 1986) can be used, or should still other groups than the free acid be desired at the C-terminus, it may be preferable to synthesize the peptide using classical methods as set forth in the Houben-Weyl text (1974).

The C-terminal amino acid, protected by Boc and by a side-chain protecting group, if appropriate, can be first coupled to a chloromethylated resin according to the procedure set forth in K. Horiki et al. (1978), using KF in DMF at about 60°C for 24 hours with stirring, when a peptide having free acid at the C-terminus is to be synthesized. Following the coupling of the BOC-protected amino acid to the resin support, the α-amino protecting group is removed, as by using trifluoroacetic acid (TFA) in methylene chloride or TFA alone. The deprotection is carried out at a temperature between about 0°C and room temperature. Other standard cleaving reagents, such as HCl in dioxane, and conditions for removal of specific α-amino protecting groups may be used as described in Schroder & Lubke (1965).

After removal of the α-amino-protecting group, the remaining α-amino- and side chain-protected amino acids are coupled step-wise in the desired order to obtain the intermediate compound defined hereinbefore, or as an alternative to adding each amino acid separately in the synthesis, some of them may be coupled to one another prior to addition to the solid phase reactor. Selection of an appropriate coupling reagent is within the skill of the art. Particularly suitable as a coupling reagent is N,N'-dicyclohexylcarbodiimide (DCC).

The activating reagents used in the solid phase synthesis of the peptides are well known in the peptide art. Examples of suitable activating reagents are carbodiimides, such as N,N'-diisopropylcarbodiimide and N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide. Other activating

reagents and their use in peptide coupling are described by Schroder & Lubke (1965) and Kapoor (1970).

Each protected amino acid or amino acid sequence is introduced into the solid-phase reactor in about a twofold or more excess, and the coupling may be carried out in a medium of dimethylformamide (DMF):CH_2Cl_2 (1:1) or in DMF or CH_2Cl_2 alone. In cases where intermediate coupling occurs, the coupling procedure is repeated before removal of the α-amino protecting group prior to the coupling of the next amino acid. The success of the coupling reaction at each stage of the synthesis, if performed manually, is preferably monitored by the ninhydrin reaction, as described by Kaiser et al. (1970). Coupling reactions can be performed automatically, as on a Beckman 990 automatic synthesizer, using a program such as that reported in Rivier et al. (1978).

After the desired amino acid sequence has been completed, the intermediate peptide can be removed from the resin support by treatment with a reagent, such as liquid hydrogen fluoride, which not only cleaves the peptide from the resin but also cleaves all remaining side chain protecting groups and also the α-amino protecting group at the N-terminus if it was not previously removed to obtain the peptide in the form of the free acid. If Met is present in the sequence, the Boc protecting group is preferably first removed using trifluoroacetic acid (TFA)/ethanedithiol prior to cleaving the peptide from the resin with HF to eliminate potential S-alkylation. When using hydrogen fluoride for cleaving, one or more scavengers such as anisole, cresol, dimethyl sulfide and methylethyl sulfide are included in the reaction vessel.

Cyclization of the linear peptide is preferably affected, as opposed to cyclizing the peptide while a part of the peptidoresin, to create bonds between Cys residues. To effect such a disulfide cyclizing linkage, fully protected peptide can be cleaved from a hydroxymethylated resin or a chloromethylated resin support by ammonolysis, as is well known in the art, to yield the fully protected amide intermediate, which is thereafter suitably cyclized and deprotected. Alternatively, deprotection, as well as cleavage of the peptide from the above resins or a benzhydrylamine (BHA) resin or a methylbenzhydrylamine (MBHA), can take place at 0°C with hydrofluoric acid (HF), followed by oxidation as described above.

Comparison of Conotoxin Classes

The present studies establish that *Conus purpurascens* venom ducts express a μ-conotoxin which has clear homology to the three previously characterized μ-conotoxins from *Conus*

geographus venom. The peptide from *Conus purpurascens*, μ-conotoxin PIIIA, like the μ-conotoxins from *Conus geographus* (Table II) is highly positively charged and has the same disulfide framework. However, of the sixteen non-cysteine amino acids in μ-conotoxin PIIIA, only five are identical in all four peptides (Arg2, Hyp8, Arg14, Lys17 and Hyp18). Some of the most strikingly divergent substitutions (Leu3 for Asp, Phe7 for Hyp) involve replacement of a hydrophilic by a hydrophobic amino acid, making μ-PIIIA significantly more hydrophobic. The conservation of Arg14, indicated by the arrow in Table II would have been predicted from structure/function studies carried out on μ-conotoxin GIIIA, which suggested that this residue was critical for biological activity. A detailed hypothesis for the placement of this residue within the vestibule of the sodium channel has been offered (Dudley et al., 1995).

Potentially, the most useful result of the present study is the difference in Na^+ channel subtype specificity of μ-conotoxin PIIIA vs. GIIIA. μ-Conotoxin PIIIA appears to target a wider spectrum of mammalian voltage-gated sodium channel subtypes than does μ-GIIIA. μ-Conotoxin PIIIA was able to displace a larger fraction of specific [^{3}H]STX binding to high affinity rat brain sites than could μ-GIIIA. However, not all [^{3}H]STX binding sites could be displaced by μ-PIIIA even at high peptide concentrations, suggesting that μ-PIIIA could discriminate between different classes of [^{3}H]STX binding sites in the mammalian central nervous system.

At the present time, voltage-gated sodium channels are primarily distinguished in situ by their tetrodotoxin sensitivity or insensitivity. The discovery and characterization of μ-conotoxin PIIIA described above provides the basis for dividing tetrodotoxin-sensitive sodium channels into three categories distinguishable by their sensitivity to μ-conotoxins:

1) Voltage-gated sodium channels which are sensitive to both μ-PIIIA and μ-GIIIA. An example of this subtype is the skeletal muscle subtype in both frog and mammalian systems. The binding data in Figure 5 are suggestive that there are central nervous system sodium channels that also fit into this category, but that they would represent only a minor fraction of the total STX/TTX-sensitive voltage-gated sodium channels present in adult rat brain.

2) A class of voltage-gated sodium channels that are sensitive to both TTX and μ-PIIIA, but which are significantly more resistant to μ-GIIIA. Figure 5 shows that rat brain Type II sodium channels belong to this category; the Type II channels may account for the binding data in Figure 4 which demonstrate that a significant fraction of the total [^{3}H]STX high affinity sites in the central nervous system are displaced by μ-PIIIA but not by μ-GIIIA.

3) Finally, both the binding data and the electrophysiology strongly suggest that a significant fraction of tetrodotoxin-sensitive sodium channels will be resistant to both μ-PIIIA and μ-GIIIA. An example of this category would be the voltage-gated sodium channels present in motor axons, which are resistant to both μ-conotoxins. The binding data indicate that a major fraction of the total CNS channels may fall into this category. In addition, the results indicate that the subtype of voltage-gated sodium channels present in motor axons must be distinct from the Type II sodium channels present in the mammalian central nervous system.

The discovery of μ-conotoxin PIIIA is also indicative that the μ-conotoxin peptide family may be broadly distributed in *Conus* species. Different μ-conotoxin sequence variants found in the about 500 species of *Conus* may be expected to exhibit different affinities for the various voltage-gated sodium channel subtypes. The situation is somewhat analogous to that found for the ω-conotoxins, where the subtype specificity of different ω-conotoxin peptides has been used to advantage to investigate the functional roles of different Ca^{2+} channel subtypes. Similarly, the μ-conotoxins should prove useful for dissecting the role of an individual Na^{+} channel subtype in a neuron, circuit or event slice preparation, particularly in those situations when multiple molecular forms of voltage-gated Na^{+} channels are present.

EXAMPLES

The present invention is described by reference to the following Examples, which are offered by way of illustration and are not intended to limit the invention in any manner. Standard techniques well known in the art or the techniques specifically described below were utilized.

EXAMPLE 1

Identification of δ-Conotoxin Peptide PVIA Sequence

Milked Venom Extraction. *C. purpurascens* specimens were collected from the Gulf of California, and venom was collected by a milking procedure. Milked venom (0.5 ml) was pooled from 50 Eppendorf tubes stored in a -70°C deep freezer. The pooled venom was kept over ice and diluted with 10 mL of 0.1% TFA in water. The solution was spun for a few minutes using a bench-top microfuge, and the supernatant was immediately subjected to

purification. There were several prominent components of the venom: when these were tested for biological activity, several of the major peaks caused flaccid paralysis if fish. Some of these components have been shown to inhibit the nicotinic acetylcholine receptor. In a pooled venom preparation, the late-eluting fractions caused paralysis and death in fish accompanied by muscle contracture and the lockjaw symptoms (rapid running, seizures and convulsions) consistent with the increased excitability in the mammalian central nervous system.

Peptide Purification by HPLC. A preparative scale reversed-phase HPLC was used for first-line purification of the milked venom. The entire 10 mL was applied to a C_{18} Vydac preparative column (22.0 x 250 mm; 20 mL/min) with a guard column (22.0 x 50.0 mm). As a secondary purification, a C_{18} Vydac analytical column (218TP54, 4.6 x 250 mm; 1 mL/min) was used. HPLC buffers were (A) 0.1% TFA in water and (B) 0.085% TFA in 90% acetonitrile. For both preparative and analytical runs, the peptides were eluted with a linear gradient of 1% buffer B increase per minute. The C_{18} analytical column was also used for purifying alkylated peptides for amino acid sequence analysis. The major components which elicit these symptoms were purified to apparent homogeneity.

Amino Acid Sequence Analysis. Peptide reduction and alkylation protocols were as described in Shon et al. (Shon et al., 1994). Reversed-phase HPLC was used for repurifying alkylated peptide. The eluted peptide was adsorbed onto Biobrene-treated glass fiber filters, and the amino acid sequence was analyzed by automated Edman degradation on an ABI Model 477A instrument. The amino acid sequence of each homogeneous peptide was determined by standard Edman degradation procedures. The amino acid sequence of each homogeneous peptide was determined by standard Edman degradation procedures. The two sequencing runs gave the identical sequence, revealing a hydrophobic 29 amino acid, which is referred to as the lockjaw peptide. The purified components were analyzed by mass spectrometry, for one component, a mass (monoisotopic MH+ = 2997.2; theoretical 2997.22) consistent with the predicted sequence from the Edman degradation with an amidated C-terminus. Together, the sequencing data and the mass spectroscopy predict the sequence Glu-Ala-Cys-Tyr-Ala-Xaa$_1$-Gly-Thr-Phe-Cys-Gly-Ile-Lys-Xaa$_2$-Gly-Leu-Cys-Cys-Ser-Glu-Phe-Cys-Leu-Pro-Gly-Val-Cys-Pro-Gly (Xaa$_1$ or Xaa$_2$ is Pro or *4-trans*-hydroxyproline) (SEQ ID NO:1). However, the other purified component did not yield a satisfactory mass spectrometric analysis. Since both components had the same

sequence, it remained a possibility that these represented forms of the peptide with free carboxyl and amidated C-termini.

EXAMPLE 2

Synthesis of δ-Conotoxin Peptide PVIA Sequence

The protected peptide resin was built using standard fmoc chemistry, couplings being carried out with equimolar amounts of amino acid derivative, DCC, and HOBT. All amino acids were purchased from Bachem (Torrence, CA), and the side chains were protected as follows: Hyp (t-Bu), Lys (boc), Ser (t-Bu), Tyr (t-Bu), Glu (t-Bu), and Thr (t-Bu). Cys residues 3, 17, 18 and 27 were protected by trt, while Cys residues 10 and 22 were protected by acm.

At the completion of synthesis, the terminal fmoc group was removed by standard treatment with piperidine/NMP (20% by volume). Peptide was removed from the resin by treatment for 2 h at 20°C with TFA/H_2O/ethanedithiol/phenol/thioanisole (90/5/2.5/7.5/5 by volume), and the whole mixture was filtered rapidly into *tert*-butyl methyl ether at -10°C. The pellet, almost free of ether, was dissolved in 60% acetonitrile containing 0.1% TFA.

A two-step oxidation protocol was used as described in Shon et al. (1994) with a few minor changes. Crude linear peptide cleaved from 100 mg resin was directly subjected to oxidation in 20 mM FeCN in 0.1 Tris-acetate buffer (pH=8.0) containing 60% acetonitrile. The peptide solution (diluted to 200 mL containing 60% acetonitrile) was dripped slowly (at least 30 min) into 200 mL of the FeCN solution in order to minimize any intermolecular disulfide bond formation. On average, 1 h is usually enough for complete oxidation. The oxidation reaction resulted in three bicyclic peptides with disulfide bonds among Cys 3, 17, 18 and 27. The three isomers were purified using a reversed-phase HPLC preparative column with a gradient of acetonitrile (27-50%) in 0.1% TFA and a flow rate of 20 mL/min. One of the three isomers gave native-like material after oxidation with 1 mM I_2 in 10% TFA and acetonitrile (5 min at room temperature, followed by a quench with 30 mM ascorbic acid).

In order to confirm the sequence assignment, and assess the functional effects of the presence or absence of C-terminal amidation, both the free and amidated forms of the peptide were synthesized and folded as described herein. Both synthetic peptides provided to be biologically active, the same *in vivo* symptomatology was induced by the synthetic forms and

the native peptide purified from venom. The earlier eluting native lockjaw peptide peak exhibits the same retention time as the synthetic amidated peptide.

EXAMPLE 3

Identification of DNA for δ-Conotoxin Peptide PVIA

A cDNA clone encoding the lockjaw peptide was purified from a library of *C. purpurascens* ω- and δ-conotoxin cDNAs using the lockjaw-specific oligonucleotide DHOG 538 (5' GARGCNTGYTAYGCNCC 3'; SEQ ID NO:3) as probe (Colledge et al., 1992). The library was constructed in the pUC vector ptz18u by cloning PCR amplicons generated using reversed-transcribed *C. purpurascens* venom duct RNA (AMV reverse transcriptase. Boehringer Mannheim) and an oligonucleotide A corresponding to the signal sequence of ω- and δ-conotoxins and oligonucleotide poly(dT) as primers. Putative clones were sequenced using the Sequenase version 2.0 DNA sequencing kit and [^{35}S]dATP (Sequences version 2.0 seventh edition protocol).

The nucleotide sequence of the clone and the inferred amino acid sequence for δ-conotoxin PVIA (lockjaw peptide) as shown in Figure 1 are set forth in SEQ ID NO:4 and SEQ ID NO:5. The open reading frame encodes a typical signal sequence, with the prepropeptide organization found in other conotoxins (Olivera et al., 1990). The precursor sequence has a typical conotoxin proteolytic cleavage site (Colledge et al., 1992; Woodward et al., 1990). The predicted mature toxin sequence is identical to the peptide purified from venom, with a C-terminal amide group. The C-terminal -Cys-Phe-Gly-Gly-OH sequence (SEQ ID NO:6) of the precursor would be processed to a -Cys-Phe-Gly-NH_2 sequence by peptidylglycine α-amidating monooxygenase (Bradbury et al., 1982; Murthy et al., 1987).

EXAMPLE 4

Assay Methods for δ-Conotoxin PVIA

Biological Assays. Goldfish (1.0-1.5 g) were injected into the intraperitoneal cavity, and 10-14 day-old Swiss Webster mice were injected intracranially (Olivera et al., 1984a). The garden snail *Helix aspersa* was injected into the head as described (Shon et al., 1994).

Electrophysiology. The cutaneus pectoris muscle of the leopard frog *Rana pipiens* was prepared and placed in a recording chamber as previously described (Yoshikami et al., 1989) except that the muscle was not pretreated with any toxins or drugs unless otherwise indicated. The motor nerve was electrically stimulated every 30 s with a rectangular suprathreshold pulse lasting 0.1 ms, and extracellular recording electrodes were used to monitor the compound action potential from the muscle.

Membrane Preparation. The crude membrane fraction was obtained from the whole brain of 6-8 month-old Sprague-Dawley rats a previously described (Cruz and Olivera, 1986).

Radiolabeling of Conotoxins. Iodination of δ-conotoxin TxVIA was carried out using the water-soluble reagent chloramine T. Two nanomoles of δ-conotoxin TxVIA dissolved in 50% acetonitrile in water was incubated for 10 min at room temperature with 2 nmol $Na^{125}I$ (1.1 mCi/nmol) and 10 nmol of chloramine T in 200 mM Tris (pH 8.6). The reaction was quenched with 50 μL of 500 mM ascorbic acid and 50 μL of 200 mM methionine, and the solution was gently extracted twice with 500 μL of diethyl ether. Upon application onto an C_{18} analytical column (Vydac), the monoiodinated TxVIA eluted shortly after the unmodified δ-conotoxin TxVIA at approximately 56% acetonitrile on a linear gradient of acetonitrile (36-63%). The label was stored as a HPLC effluent at -20°C with 57 mM methionine and centrifuged before use in binding assays. ω-Conotoxin GVIA was labeled by resuspending 10 nmol of peptide in 0.1% TFA, adding an equal volume of 0.25 M Tris-HCl, pH 7.0, and incubating with an equivalent amount of chloramine T and 4 nmol of $Na^{125}I$ (2.2 mCi/nmol) for 10 min at room temperature. The [^{125}I]ω-GVIA was purified by HPLC as previously described (Cruz and Olivera, 1986; Cruz et al., 1987).

Competitive binding Assays. Two assay procedures were used. The first was optimized for [^{125}I]δ-conotoxin TxVIA binding (Hillyard et al., 1992). The second conditions were standard ω-conotoxin binding assays (Hillyard et al., 1992), modified by adding 130 mM NaCl, 5 mM $CaCl_2$, 1.3 mM KCl and 0.8 mM $MgCl_2$ to the assay mix.

EXAMPLE 5

Biological Activity of δ-Conotoxin Peptide PVIA

Electrophysiology. The effects of the lockjaw peptide on a frog neuromuscular junction preparation were examined. Clearly, neuromuscular transmission was not blocked by the lockjaw peptide; instead trains of action potentials were produced in response to a single nerve stimulus when the preparation was exposed to toxin. The effects of the toxin were reversible. The experiment was performed six times and in every case repetitive action potentials were observed. The number of action potentials produced by stimulation increased with time of exposure to the toxin. The results provide a plausible explanation for the fish lockjaw syndrome; in effect, repetitive action potentials in the fish jaw musculature elicited by the lockjaw peptide would result in a titanic paralysis and the rigid extension of the fish mouth part.

Competitive Binding with ω-Conotoxin GVIA. The arrangement of cysteine residues in the lockjaw peptide is characteristic of the so-called "four-loop" family of *Conus* peptides. In fish-hunting cones, the major peptide family with the four-loop Cys motif is the ω-conotoxins, which inhibit voltage-sensitive Ca channels (Myers et al., 1993). Indeed, when the sequence of the lockjaw peptide precursor is aligned with the precursor sequence of an ω-conotoxin, considerable identity is observed (Figure 1). For this reason, the purified lockjaw peptide was tested for binding to the ω-conotoxin site. In a competition binding experiment using ^{125}I-radiolabeled GVIA, no displacement of specific ω-conotoxin binding (see Table 1) was found, suggesting that the lockjaw peptide is not a member of the ω-conotoxin family.

TABLE 1

Binding Competition Experiments

	[^{125}I]δ-TxVIA label (cpm bound)	[^{125}I]ω-GVIA label (cpm bound)
no additions	4890 ± 691	4405 ± 123
δ-Conotoxin TxVIA	925 ± 39	4719 ± 40
ω-conotoxin GVIA	4710 ± 450	954 ± 209
δ-conotoxin PVIA (amidated)	889 ± 92	4927 ± 253
δ-conotoxin PVIA (nonamidated)	848 ± 196	4393 ± 458

Binding Evidence That the Lockjaw Peptide is a δ-Conotoxin. The *C. purpurascens* peptide exhibits an even greater similarity to the precursor sequence of a previously characterized peptide from a snail-hunting Conus venom, δ-conotoxin TxVIA (Woodward et al., 1990). The δ-conotoxins were previously shown to bind specifically to voltage sensitive Na channels (Fainzilber et al., 1994), causing a delay in channel inactivation resulting in an increase in Na conductance (Hasson et al., 1993; Shon et al., 1994). The sequence homology in Figure 1 strongly suggested that the lockjaw peptide might be a member of the δ-conotoxin family.

In order to confirm whether the *C. purpurascens* peptide was in fact a δ-conotoxin, binding competition was performed using radiolabeled δ-conotoxin TxVIA as the probe for high-affinity sites on rat brain Na channel (Fainzilber et al., 1994). The results are shown in Table 1 above. It is clear that the peptide completely displaced specific δ-conotoxin TxVIA binding under assay conditions where there was not detectable displacement of ω-conotoxin GVIA binding. The experiments in Table 1 were carried out under conditions optimal for δ-conotoxin TxVIA binding; even under assay conditions optimal for ω-conotoxin GVIA binding, the lockjaw peptide displaced the δ-conotoxin but not [^{125}I]ω-GVIA. These results, together with the precursor sequence homologies, support the conclusion that the *C. purpurascens* peptide targets to the δ-conotoxin binding site on Na channels and is not an ω-conotoxin. The initially seen electrophysiological effects are thus rationalized by the lockjaw peptide increasing voltage-gated Na^+ currents (Fainzilber et al., 1991), thereby making the neuromuscular junction more electrically excitable.

In vivo Experiments. The molecular genetic and binding data which indicate that the lockjaw peptide is a δ-conotoxin and not an ω-conotoxin are reinforced by the observed *in vivo* biological activity of the peptide on fish and mice. The ω-conotoxins cause a characteristic shaking syndrome when injected intracranially into mice. In contrast, injection of 0.5 nmol of the purified lockjaw peptide caused hyperactivity, rapid running, limb extension, and death. At higher doses (≈ 5 nmol), the peptide was remarkably toxic in mice, causing death in 10 s. Thus, the peptide is a potent excitotoxin in mammals, a result consistent with Na channel-targeted ligand, which increases conductance, rather than a calcium channel blocker of the δ-conotoxin class.

In fish, the peptide elicited spurts of rapid swimming, with twisted motions, quivering fins, and the lockjaw extended mouth syndrome. Rigid paralysis and death were observed if 0.5-5.0 nmol was injected.

Previously characterized δ-conotoxins were highly potent in all molluscs tested (Hillyard et al., 1989; Fainzilber et al., 1991; Fainzilber et al., 1994; Shon et al., 1994). The peptide from *C. purpurascens* was injected into a mollusc. In contrast to the results with δ-conotoxins GmVIA and TxVIA which cause typical "King-Kong type" symptomatology (Hillyard et al., 1989) in this snail, the lockjaw peptide elicited no detectable biological effects. Thus, the lockjaw peptide is a potent toxin in vertebrate systems but is inactive in this mollusc.

EXAMPLE 6

Identification of a cDNA Clone from *Conus purpurascens* Encoding a Putative μ-Conotoxin

The feasibility of discovering new *Conus* peptides from the predicted amino acid sequences encoded by cDNA clones was previously demonstrated with ω-conotoxins (Hillyard et al., 1992). The method involves preparing cDNA libraries and screening these with mixed oligonucleotides in a manner similar to that described by Hillyard et al. (1992). A cDNA library was prepared using mRNA from the venom duct of *C. purpurascens* as previously described (Woodward et al., 1990; Colledge et al., 1992) and this library was subsequently screened using mixed oligonucleotides to identify clones putatively encoding conotoxins. Many positive clones were identified and sequenced. Several of these cDNA clones contained the nucleotide sequence shown in Figure 2. The predicted amino acid sequence form this nucleotide sequence strongly suggested that the clones might encode a μ-conotoxin. Despite the significant sequence divergence from previously characterized μ-conotoxins (μ-GIIIA, μ-GIIIB and μ-GIIIC from *Conus geographus*) there were a number of important features similar to these earlier characterized conotoxins. These similarities include: the pattern of Cys residues, the high net positive charge, and the apparent conservation of the critical Arg residue (residue 14 of the predicted mature peptide) believed to be essential for μ-conotoxin function (Sato et al., 1991; Becker et al., 1992).

EXAMPLE 7

Synthesis of μ-Conotoxin Peptide PIIIA Sequence

The predicted 22-residue peptide, including post-translational modifications modeled on other related peptides was chemically synthesized. The post-translational modifications include changing Gln1 to pyroglutamate, prolines to hydroxyproline, the C-terminal Cys-Cys-Gly-Arg (SEQ ID NO: 7) to Cys-Cys-NH_2. The resulting peptide, with the disulfide bonding indicated, is referred to as μ-conotoxin PIIIA (μ-PIIIA) based on the physiological evidence detailed below. The structure is:

SEQ ID NO: 2

where Z = pyroglutamate and O = 4-trans-hydroxyproline. The pure synthetic peptide caused flaccid paralysis in both mice and fish, as expected for a μ-conotoxin.

The peptide was built in two stages based on the linear sequence predicted from the cDNA isolate. First, the protected peptide resin minus the N-terminal pyroglutamate was built by standard Fmoc chemistry on an ABI model 477A peptide synthesizer. Pyroglutamate was then added manually to some of the resin to produce the complete peptide. After cleavage from the respective resins, the linear peptides ([1-22] and [2-22]) were purified by preparative reversed phase HPLC. Disulfide bridges were allowed to form in the presence of a glutathione redox buffer, and the products were again fractionated by preparative HPLC. The major oxidation products in each case were obtained in highly purified form.

Peptide bond coupling was carried out with equimolar amounts of amino acid derivative, DCC and HOBT, and the terminal Fmoc group was removed by treatment with piperidine/NMP (20% by volume). The side chain Fmoc-protected amino acids were purchased from Bachem (Torrance, CA): these are Hyp (t-Bu), Lys (Boc), Ser (t-Bu), Arg (pmc), Gln (trt), His (trt) and Cys (trt). In the second stage, pyroglutamic acid was manually coupled to the peptide resin. Pyroglutamic acid (0.25 mmol; Sigma) was activated in 1 ml solution of 1 M DICC/1 M HOBT in NMP for 30 minutes, and the solution was added to 100 mg (0.012 mmol) peptide resin. The reaction mixture was stirred for 2.5 hours and centrifuged. The resin was then washed with NMP

five times, followed by three washes with methanol. Because the pyroglutamate was not protected, the removal of an Fmoc group was not necessary. The final resin was dried, and subjected to peptide cleavage as described previously (Shon et al., 1995). The cleavage mixture was filtered into tert-butyl methyl ether at -10°C. Peptides immediately precipitated, and the solution was centrifuged to separate the pellet, which was washed three times with the ether.

The pellet was dissolved in 60% acetonitrile containing 0.1% TFA, and purified by reversed phase HPLC. Several runs were required on both preparative and semi-preparative columns to obtain pure linear peptide. The glutathione oxidation protocol previously described (Hopkins et al., 1995) was used to oxidize the linear peptide. The major peak from overnight oxidation was repurified on both preparative and semi-preparative columns.

To obtain the unblocked analog μ-PIIIA[2-22], the same cleavage and oxidation procedures were carried through on a sample of resin before the pyroglutamate was added.

EXAMPLE 8

Iodination of μ-Conotoxin PIIIA[2-22]

The peptide solution (5-10 nmol) in sodium phosphate buffer (0.25 M, pH 7.5) (about 0.4 ml) was incubated with an equal volume of 2 mM I_2 dissolved in methanol, for 10 minutes at ambient temperature. The reaction mixture was quenched with ascorbic acid, and then subjected to reversed phase HPLC. With this incubation time, most of the product was the di-iodinated peptide. Shorter incubations were used to prepare the mono-iodinated derivative.

EXAMPLE 9

Disulfide Bridge Analysis

Disulfide bridge analysis was carried out on two analogs (the peptide without an N-terminal pyroglutamate, and the same peptide with the His residue di-iodinated). The disulfide connectivity of μ-PIIIA[2-22] was analyzed by the partial reduction strategy of Gray (Gray, 1993), and found to be the same as that of the known μ-conotoxins from *C. geographus*. The shorter analog was chosen for analysis, because the terminal pyroglutamate of the full-length peptide greatly complicates sequencing. Partial reduction with TCEP gave intermediates that were not well

resolved from fully reduced or fully oxidized peptides, and only one suitable product could be isolated. This was labeled with iodoacetamide, then reduced and further labeled with 4-vinylpyridine; the pattern of labeling showed that it represented an intermediate with a single disulfide between Cys3 (residue 11) and Cys6 (residue 22).

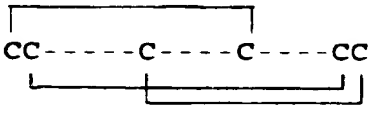
The analysis was completed using the monoiodohistidine derivative of μ-PIIIA[2-22], which gave three intermediates that were shifted away from the fully reduced and fully oxidized peptides. One of these proved to have a single disulfide between Cys2 (residue 4) and Cys5 (residue 21); a second had been reduced only at the Cys3 - Cys6 bridge; the third, though not sequenced completely, appeared to be analogous to the product obtained form the non-iodinated peptide. Thus, all results are consistent with a disulfide connectivity exactly equivalent to that of the μ-GIII series of toxins (Cys1-Cys4; Cys2-Cys5; Cys3-Cys6). The sequences and disulfide connectivity of all known μ-conotoxins are shown in Table II.

Table II

Comparison of Sequences of Known μ-Conotoxins

	↓	
μ-PIIIA	ZRLCCGFOXSCRSRQCKOHRCC*	SEQ ID NO: 2
	↓	
μ-GIIIA	RDCCTOOKKCKDRQCKOQRCCA*	SEQ ID NO: 8
μ-GIIIB	RDCCTOORKCKDRRCKOMKCCA*	SEQ ID NO: 9
μ-GIIIC	RDCCTOOKKCKDRRCKOLKCCA*	SEQ ID NO: 10

Disulfide Bonding



Z = pyroglutamate; O = 4-trans-hydroxyproline

Arrow (↓) indicates the conserved Arg.

* indicates an amidated carboxy terminus; the amidation for GIIIC was not directly determined experimentally, but is inferred by homology.

EXAMPLE 10

Electrophysiology

The effects of the peptide on frog muscle (cutaneus pectoralis) were investigated. A sketch of the recording chamber is shown in Figure 3A. Current was injected into the muscle across partition 1; the recording electrodes monitored the potential across partition 3. Partition 2 served to electrically isolate the recording from the stimulating electrodes. Toxin was added only into the compartment D.

The cutaneus pectoris muscle from ~7 cm Rana pipiens frogs was used. The muscle was trimmed longitudinally so that only the lateral one-quarter of muscle remained (cf. Yoshikami et al., 1989). The trimmed muscle was pinned flat on the floor of a shallow trough (~4 mm x 16 mm x 1 mm deep) fabricated from Sylgard (a silicone elastomer, Dow Chemical Co.). The trough had four transverse slits cut into its wall by a razor blade (see Figure 3A). Thus, once the muscle was pinned in place, the trough could be partitioned into four compartments by inserting a 0.1 mm thick Mylar sheet into each slot. To prevent the Mylar partitions from cutting into the muscle pinned to the floor of the trough, two strips of Mylar (~1 mm wide x 15 mm long x 0.1 mm thick) were placed on either side of the muscle to serve as stops. All four compartments contained Ringer's solution. Stimulating electrodes were located in the first two compartments (A and B), and recording electrodes were located in the second two compartments (C and D). A ground electrode was located in compartment B. All electrodes were bare Pt wires. The recording electrode in C was connected to the negative input, and that in D to the positive input, of a differential AC preamplifier. The stimulating electrodes were connected to a stimulus isolation unit, and supramaximal, 1 ms-long rectangular pulses were used to directly elicit action potentials in the muscle. Stimuli were applied at a frequency of 1/minute or less. When the action potential propagated into chamber C, a positive response was recorded by the preamplifier, and the further propagation of the action potential into chamber D was registered by the preamplifier as a negative response. Thus, the extracellularly recorded action potential from the population of fibers in the muscle was recorded as a biphasic response, with the phases separated from each other by only a few milliseconds (see Figure 3B). To examine the effect of the toxin, the plain Ringer's solution in chamber D was replaced by one containing toxin. If the toxin blocked sodium channels, attenuation of only the late negative phase should be observed.

The early positive phase should remain largely unaltered reflecting the fact that portions of the muscle not exposed to toxin remained normal. Thus, there are two advantages of exposing only the solution in chamber D to toxin. One, this allows the response in chamber C to serve as an internal control for the overall vitality of the muscle preparation as well as to insure that the stimulus remained supramaximal; and two, the volume of toxin solution necessary is reduced, in these experiments 25 µl sufficed.

A control response before toxin addition is shown in Figure 3B. The progression of the action potential between segments C and D is readily apparent; the biphasic waveform generated represents propagation of the action potential from C to D. The peaks of the responses as a function of time before, during, and after toxin addition are shown in Figure 3C. With the toxin added to segment D, the action potential clearly propagated into segment C, causing the voltage change characteristic of the first half of the biphasic waveform in Figure 3B; however, the negative phase was completely abolished, indicating that although a normal action potential was generated, transmission in segment D of the muscle was abolished. These results are consistent with inhibition of voltage-gated sodium channels on the muscle plasma membrane. Even upon washing for many hours in the absence of toxin, no recovery was observed in segment D (see Figure 3C), although action potential propagation to segment C was essentially normal (a slight rundown was observed with time). Similar results were also observed with the µPIIIA[2-22] analog of the toxin (results not shown). During exposure to toxin, the amplitude of the positive phase increased somewhat while that of the negative phase was abolished as is also evident in Figure 3C. Upon washout of toxin, the amplitude of the negative phase remained nil while that of the positive phase continue to increase slightly for about 10 minutes before slowly and continuously decreasing over the next 4 hours. This decline continued at essentially a constant rate for the next 13 hours (not shown) at which time the response was ~10 mV, and the experiment was terminated. These experiments show that the negative phase is completely, and irreversibly obliterated by exposure to toxin, whereas the positive phase remains largely intact indicating that no untoward systemic changes occurred. Upon exposure to toxin, the positive phase initially becomes larger because the counteracting negative phase becomes smaller. The initial rising phase of the positive phase is also slightly delayed following exposure to toxin; this is thought to be due to leakage of the toxin into compartment C with an attendant decrease in the propagation velocity of the action potential in that compartment. Leak of toxin into

compartment C is also thought to be responsible for the decrement in the amplitude of the positive phase as well as delayed time to peak observed in the response taken >4.5 hours later.

Nerve-muscle preparations were also examined; when the motor nerve was electrically stimulated, a muscle action potential was recorded and a muscle twitch observed. When the entire nerve-muscle preparation was exposed to toxin, muscle twitching and action potentials were completely abolished; however, excitatory post-synaptic responses were still recorded (results not shown). Thus, propagation of action potentials in the motor axon is not blocked, unlike action potential propagation in muscle.

The results are consistent with the activity of a μ-conotoxin; the homologous peptides from *Conus geographus* have previously been shown to be highly specific for the skeletal muscle Na^+ channel subtype in peripheral systems. Although μ-GIIIA and μ-PIIIA selectively inhibit skeletal muscle action potentials, a notable difference is that the latter peptide appears to act much more irreversibly in the frog neuromuscular preparation. The new peptide should be the most convenient pharmacological agent available for irreversibly preventing muscle twitching when the synaptic electrophysiology of amphibian neuromuscular junctions is investigated.

EXAMPLE 11

Binding Competition Experiments

In order to further establish that the peptide is a μ-conotoxin, binding displacement experiments were performed with [^{3}H]saxitoxin as the radiolabeled ligand, and *Electrophorus electricus* electric organ membranes as the source of receptors (Figure 4). [^{3}H]Saxitoxin binding to rat brain membranes was carried out by the protocol of Doyle et al. (1993) except that the assays were scaled down to a volume of 0.25 ml and 1 mM PMSF, 1 μM leupeptin and 1 μM pepstatin were present. Electric eel membranes were prepared as described by Becker et al. (1989) except that the homogenizing buffer used was 10 mM HEPES-Tris, 10 mM EDTA, 10 mM EGTA, 1 mM PMSF, 1 μM leupeptin and pepstatin, pH 7.0.

As expected, μ-conotoxin PIIIA displaces [^{3}H]saxitoxin binding to electric organ membranes, which contain a high density of the skeletal muscle subtype of voltage-gated sodium channels.

Clearly, μ-conotoxin PIIIA has a high affinity ($K_D \sim 3 \times 10^{-9}$ M) for the saxitoxin binding site in the electric organ.

Somewhat surprisingly, it was found that μ-PIIIA also displaced a significant fraction (>50%) of specific [^{3}H]saxitoxin binding to crude membranes from rat brain. A comparison of μ-PIIIA and μ-GIIIA displacement of specific [^{3}H]saxitoxin binding to rat brain sites is shown in Figure 4. These data indicate that μ-PIIIA displaces more than half of the [^{3}H]saxitoxin high affinity sites in rat brain (apparent $K_D \sim 30$ nM); in contrast, μ-GIIIA displaced about 20% of specific [^{3}H]saxitoxin binding at the same concentrations. These results suggest that there are μ-PIIIA-sensitive, μ-GIIIA-resistant Na^+ channels in the mammalian CNS.

EXAMPLE 12

Central Nervous System (CNS) Electrophysiology

In order to establish directly whether μ-conotoxin PIIIA could affect voltage-gated sodium channels in the CNS, the effect of the toxin was tested on a major subtype of voltage-gated sodium channels found in central neurons, the Type II voltage-gated sodium channels (Figures 5A-5C). Oocytes from *Xenopus laevis* were prepared as described previously (Stühmer, 1992). mRNA encoding rat Type II sodium channel α-subunit (Noda et al., 1986) was injected into stage VI oocytes (30-50 ng/oocyte). The vitelline membranes of the oocytes were removed mechanically with fine forceps and currents were recorded 2-6 days after injection under two-electrode voltage clamp control with a Turbo-Tec amplifier (NPI Elekronik, Tamm, Germany) driven by the Pulse+PulseFit software package (HEKA Electronik, Lambrecht, Germany). Intracellular electrodes were filled with 2 M KCl and had a resistance between 0.6 and 0.8 MΩ. Current records were low-pass filtered at 3 kHz and sampled at 10 kHz. The bath solution was normal frogs Ringer's (NFR) containing (in mM): 115 NaCl, 2.5 KCl, 1.8 $CaCl_2$, 10 Hepes pH 7.2 (NaOH). Leak and capacitive currents were corrected on-line by using a P/n method. Toxin solution was prepared in NFR added to the bath chamber.

The Type II voltage-gated sodium channels were previously shown to be TTX-sensitive but resistant to 1-2 μM of μ-conotoxin GIIIA (Terlau et al., 1996; Noda et al., 1986). μ-PIIIA blocked type II Na^+ channels from rat expressed in *Xenopus* oocytes (Noda et al., 1986); the presence of μ-PIIIA (2 μM) in the bath solution abolished nearly all Na^+ currents (Figure 5B), but in a reversible

manner (Figure 5C). Thus, rat brain Type II Na^+ channels apparently belong to the TTX- and μ-PIIIA-sensitive, but μ-GIIIA resistant class of Na^+ channels in the mammalian CNS.

It will be appreciated that the methods and compositions of the instant invention can be incorporated in the form of a variety of embodiments, only a few of which are disclosed herein. It will be apparent to the artisan that other embodiments exist and do not depart from the spirit of the invention. Thus, the described embodiments are illustrative and should not be construed as restrictive.

LIST OF REFERENCES

Becker, S. et al. (1989). Synthesis and characterization of μ-conotoxin IIIA. *Eur. J. Biochem.* **185**:79-84.

Becker, S. et al. (1992). Action of derivatives of μ-conotoxin GIIIA on sodium channels. Single amino acid substitutions in the toxin separately affect association and dissociation rates. *Biochemistry* **31**:8229-8238.

Bodansky et al. (1966). *Chem. Ind.* **38**:1597-98.

Bradbury, A.F., et al. (1982). *Nature* **298**:686-688.

Burns, J.A. et al. (1991). Selective reduction of disulfides by tris-[2-carboxyethyl]phosphine. *J. Org. Chem.* **56**:2648-50.

Catterall, W.A. (1992). Cellular and molecular biology of voltage-gated sodium channels. *Physiol. Rev. Supp.* **72**:S15-S48.

Colledge, C.J. et al. (1992). Precursor structure of ω-conotoxin GVIA determined from a cDNA clone. *Toxicon* **30**:1111-1116.

Cruz, L.J. et al. (1976). Block of P-type calcium channels by the funnel web spider toxin w-Aga-IVA.. *Nature* **355**:827-29.

Cruz, L.J. et al. (1985). *Conus geographus* toxins that discriminate between neuronal and muscle sodium channels. *J. Biol. Chem.* **260**:9280-9288.

Cruz, L.J. and Olivera, B.M. (1986). *J. Biol. Chem.* **361**:6230-6233.

Cruz, L.J., et al. (1987). In *Integration and Control of Metabolic Processes. Pure and Applied Aspects*, Kon, O.L., ed.. Cambridge University Press, Cambridge, pp. 95-102.

Doyle, D.D. et al. (1993). Divalent cation competition with [^{3}H]saxitoxin binding to tetrodotoxin-resistant and -sensitive sodium channels. *J. Gen. Physiol.* **101**:153-182.

Dudley, S.C. et al. (1995). A μ-Conotoxin-Insensitive Na^+ Channel Mutant: Possible Localization of a Binding Site at the Outer Vestibule. *Biophys. J.* **69**:1657-1665.

Fainzilber, M., et al. (1991). *Eur. J. Biochem.* **202**:589-595.

Fainzilber, M., et al. (1994). *Biochemistry* **33**:9523-9529.

Fainzilber, M., et al. (1994). *J. Biol. Chem.* **269**:2574-2580.

Fox, A.P. et al. (1987). *J. Physiol. Lond.* **394**:149-172.

Gray, W.R. (1993). Disulfide Structures of Highly Bridged Peptides: A New Strategy for Analysis. *Protein Science* **2**:1732-1748.

Hasson, A. et al. (1993). *Eur. J. Neurosci.* **5**:56-64.

Hillyard, D.R. et al. (1989). *Biochem.* **28**:358.

Hillyard, D.R. et al. (1992). A new Conus peptide ligand for mammalian presynaptic Ca^{2+} channels. *Neuron* **9**:69-77.

Hopkins, C. et al. (1995). A new family of *Conus* peptides targeted to the nicotinic acetylcholine receptor. *J. Biol. Chem.* **270**:22361-22367.

Horiki, K. et al. (1978). *Chemistry Letters* 165-68.

Kaiser et al. (1970). *Anal. Biochem.* **34**:595.

Kapoor (1970). *J. Pharm. Sci.* **59**:1-27.

Kobayashi, J., et al. (1982). *Biochem. Biophys. Res. Commun.* **105**:1389-1395.

Kornreich, W.D. et al. (1986). U.S. Patent No. 4,569,967.

McIntosh, J.M. et al. (1994). *J. Biol. Chem.* **269**:16733-16739.

Methoden der Organischen Chemie (Houben-Weyl): Synthese von Peptiden, E. Wunsch (Ed.). Georg Thieme Verlag, Stuttgart, Ger. (1974).

Miljanich, G.P. et al. (1993). U.S. Patent No. 5,264,371.

Murthy, A.S.N., et al. (1987). *Mol. Endocrinol.* **1**:290-299.

Myers, R.A. et al. (1993). *Conus* Peptides as Chemical Probes for Receptors and Ion Channels. *Chem. Rev.* **93**:1923-1936.

Narahashi, T. et al. (1964). Tetrodotoxin blockage of sodium conductance increase in lobster giant neurons. *J. Gen. Physiol.* **47**:965-974.

Noda, M. et al. (1986). Existence of distinct sodium channel messenger RNAs in rat brain. *Nature* **320**:188-191.

Olivera, B.M. et al. (1984). U.S. Patent 4,447,356.

Olivera, B.M. et al. (1984a). *Biochem.* **23**:5087.

Olivera, B.M. et al. (1985). Peptide neurotoxins from fish-hunting cone snails. *Science* **230**:1338-1343.

Olivera, B.M. et al. (1990). Diversity of *Conus* neuropeptides. *Science* **249**:257-263.

Olivera, B.M. et al. (1994). Calcium channel diversity and neurotransmitter release: The ω-conotoxins and ω-agatoxins. *Ann. Rev. Biochem.* **63**:823-867.

Rivier, J.R. et al. (1978). *Biopolymers* **17**:1927-38.

Sambrook, J. et al. (1979). *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Sato, S. et al. (1983). The amino acid sequences of homologous hydroxyproline containing myotoxins from the marine snail *Conus geographus* venom. *FEBS Lett.* **155**:277-280.

Sato, K. et al. (1991). Active site μ-conotoxin GIIIA, a peptide blocker of muscle sodium channels. *J. Biol. Chem.* **266**:16989-16991.

Schacher, S. and Proshansky, E. (1983). *J. Neurosci.* **3**:2403.

Schroder & Lubke (1965). *The Peptides* **1**:72-75, Academic Press, NY.

Shon, K.-J., et al. (1994). *Biochemistry* **33**:11420-11425.

Shon, K. et al. (1995). Purification, characterization and cloning of the lockjaw peptide from *Conus purpurascens* venom. *Biochemistry* **34**:4913-4918.

Spira, M.E. et al. (1993). *Isr. J. Med. Sci.* **29**:530-543.

Stewart and Young, *Solid-Phase Peptide Synthesis*, Freeman & Co., San Francisco, CA (1969).

Stone, B.L. and Gray, W.R., (1982). Occurrence of hydroxyproline in a toxin from the marine snail *Conus geographus*. *Arch. Biochem. Biophys.* **216**:756-767.

Stühmer, W. (1992). Electrophysiological recordings from *Xenopus* oocytes. *Meth. Enzymol.* **207**:319-339.

Terlau, H. et al. (1996). μO-Conotoxin MrVIA inhibits mammalian sodium channels but not through Site I. *J. Neurophysiol.*, submitted.

Vale et al. (1978). U.S. Patent 4,105,603.

Woodward, S.R. et al. (1990). Constant and hypervariable regions in conotoxin propeptides. *EMBO J.* **1**:1015-1020.

Yoshikami. D. et al. (1989). The inhibitory effects of omega-conotoxins on calcium channels and synapses. *Ann. N.Y. Acad. Sci.* **560**:230-248.

U.S. Patent 3,972,859 (1976).

U.S. Patent 3,842,067 (1974).

U.S. Patent 3,862,925 (1975).

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Shon, Ki-Joon
Grilley, Michelle M.
Olivera, Baldomero M.
Yoshikami, Doju
Marsh, Maren
Cruz, Lourdes J.
Hillyard, David R.

(ii) TITLE OF INVENTION: Conotoxin Peptides

(iii) NUMBER OF SEQUENCES: 14

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Venable, Baetjer, Howard & Civiletti, LLP
(B) STREET: 1201 New York Avenue, N.W., Suite 1000
(C) CITY: Washington
(D) STATE: DC
(E) COUNTRY: U.S.A.
(F) ZIP: 20005

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: WO
(B) FILING DATE: 17-APR-1996
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/423,561
(B) FILING DATE: 17-APR-1995

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Saxe, Stephen A.
(B) REGISTRATION NUMBER: 38,609
(C) REFERENCE/DOCKET NUMBER: 24260-107674

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 202-962-4848
(B) TELEFAX: 202-962-8300

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Conus purpurascens

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 6
(D) OTHER INFORMATION: /note= "Xaa at residue 6 is Pro or 4-trans-hydroxyproline"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 14
(D) OTHER INFORMATION: /note= "Xaa at residue 14 is Pro or 4-trans-hydroxyproline"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Glu Ala Cys Tyr Ala Xaa Gly Thr Phe Cys Gly Ile Lys Xaa Gly Leu
1 5 10 15

Cys Cys Ser Glu Phe Cys Leu Pro Gly Val Cys Pro Gly
20 25

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Conus purpurascens

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 1
(D) OTHER INFORMATION: /product= "OTHER"
/note= "Amino acid 1 is pyroglutamate or glutamine."

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 8
(D) OTHER INFORMATION: /product= "OTHER"
/note= "Amino acid 8 is 4-trans-hydroxyproline or proline."

(ix) FEATURE:
(A) NAME/KEY: Disulfide-bond
(B) LOCATION: 4..16

(ix) FEATURE:
(A) NAME/KEY: Disulfide-bond
(B) LOCATION: 5..21

(ix) FEATURE:
(A) NAME/KEY: Disulfide-bond
(B) LOCATION: 11..22

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 18
(D) OTHER INFORMATION: /product= "OTHER"
/note= "Amino acid 18 is 4-trans-hydroxyproline or proline."

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 22
(D) OTHER INFORMATION: /product= "OTHER"
/note= "The carboxy terminus may be amidated."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Xaa Arg Leu Cys Cys Gly Phe Xaa Lys Ser Cys Arg Ser Arg Gln Cys
1 5 10 15

Lys Xaa His Arg Cys Cys
20

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "DNA PRIMER"

(iii) HYPOTHETICAL: YES

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Conus purpurascens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GARGCNTGYT AYGCNCC 17

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 243 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Conus purpurascens

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..243

(ix) FEATURE:
(A) NAME/KEY: sig_peptide
(B) LOCATION: 1..66

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 154..243

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
ATG AAA CTG ACG TGC GTG ATG ATC GTT GCT GTG CTG TTC TTG ACT GCC                48
Met Lys Leu Thr Cys Val Met Ile Val Ala Val Leu Phe Leu Thr Ala
-51 -50                 -45                 -40

TGG ACA TTC GTC ACG GCT GAT GAC TCC AAA AAT GGA CTG GAG AAT CAT               96
Trp Thr Phe Val Thr Ala Asp Asp Ser Lys Asn Gly Leu Glu Asn His
-35                 -30                 -25                 -20

TTT TGG AAG GCA CGT GAC GAA ATG AAG AAC CGC GAA GCC TCT AAA TTG              144
Phe Trp Lys Ala Arg Asp Glu Met Lys Asn Arg Glu Ala Ser Lys Leu
                -15                 -10                 -5

GAC AAA AAG GAA GCC TGC TAT GCG CCT GGT ACT TTT TGT GGC ATA AAG              192
Asp Lys Lys Glu Ala Cys Tyr Ala Pro Gly Thr Phe Cys Gly Ile Lys
        1                   5                   10

CCC GGG CTA TGC TGC AGT GAG TTT TGT CTC CCG GGC GTC TGC TTC GGT              240
Pro Gly Leu Cys Cys Ser Glu Phe Cys Leu Pro Gly Val Cys Phe Gly
    15                  20                  25

GGT                                                                          243
Gly
 30
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 81 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
Met Lys Leu Thr Cys Val Met Ile Val Ala Val Leu Phe Leu Thr Ala
-51 -50                 -45                 -40

Trp Thr Phe Val Thr Ala Asp Asp Ser Lys Asn Gly Leu Glu Asn His
-35                 -30                 -25                 -20

Phe Trp Lys Ala Arg Asp Glu Met Lys Asn Arg Glu Ala Ser Lys Leu
                -15                 -10                  -5

Asp Lys Lys Glu Ala Cys Tyr Ala Pro Gly Thr Phe Cys Gly Ile Lys
              1               5                  10

Pro Gly Leu Cys Cys Ser Glu Phe Cys Leu Pro Gly Val Cys Phe Gly
     15                  20                  25

Gly
 30
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: C-terminal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Conus purpurascens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
Cys Phe Gly Gly
1
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: C-terminal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Conus purpurascens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Cys Cys Gly Arg
1

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Conus geographus

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 6
(D) OTHER INFORMATION: /product= "4Hyp"
/note= "Amino acid 6 is 4-trans-hydroxyproline."

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 7
(D) OTHER INFORMATION: /product= "4Hyp"
/note= "Amino acid 7 is 4-trans-hydroxyproline."

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 17
(D) OTHER INFORMATION: /product= "4Hyp"
/note= "Amino acid 17 is 4-trans-hydroxyproline."

(ix) FEATURE:
(A) NAME/KEY: Disulfide-bond
(B) LOCATION: 3..15

(ix) FEATURE:
(A) NAME/KEY: Disulfide-bond
(B) LOCATION: 4..20

(ix) FEATURE:
(A) NAME/KEY: Disulfide-bond
(B) LOCATION: 10..21

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 22
(D) OTHER INFORMATION: /product= "OTHER"
/note= "The carboxy terminus is amidated."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Arg Asp Cys Cys Thr Xaa Xaa Lys Lys Cys Lys Asp Arg Gln Cys Lys
1 5 10 15

Xaa Gln Arg Cys Cys Ala
20

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Conus geographus

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 6
(D) OTHER INFORMATION: /product= "4Hyp"
/note= "Amino acid 6 is 4-trans-hydroxyproline."

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 7
(D) OTHER INFORMATION: /product= "4Hyp"
/note= "Amino acid 7 is 4-trans-hydroxyproline."

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 17
(D) OTHER INFORMATION: /product= "4Hyp"
/note= "Amino acid 17 is 4-trans-hydroxyproline."

(ix) FEATURE:
(A) NAME/KEY: Disulfide-bond
(B) LOCATION: 3..15

(ix) FEATURE:
(A) NAME/KEY: Disulfide-bond
(B) LOCATION: 4..20

(ix) FEATURE:
(A) NAME/KEY: Disulfide-bond
(B) LOCATION: 10..21

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 22
(D) OTHER INFORMATION: /product= "OTHER"
/note= "The carboxy terminus is amidated."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Arg Asp Cys Cys Thr Xaa Xaa Arg Lys Cys Lys Asp Arg Arg Cys Lys
1 5 10 15

Xaa Met Lys Cys Cys Ala
20

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Conus geographus

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 6
(D) OTHER INFORMATION: /product= "4Hyp"
/note= "Amino acid 6 is 4-trans-hydroxyproline."

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 7
(D) OTHER INFORMATION: /product= "4Hyp"
/note= "Amino acid 7 is 4-trans-hydroxyproline."

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 17
(D) OTHER INFORMATION: /product= "4Hyp"
/note= "Amino acid 17 is 4-trans-hydroxyproline."

(ix) FEATURE:
(A) NAME/KEY: Disulfide-bond
(B) LOCATION: 3..15

(ix) FEATURE:
(A) NAME/KEY: Disulfide-bond
(B) LOCATION: 4..20

(ix) FEATURE:
(A) NAME/KEY: Disulfide-bond
(B) LOCATION: 10..21

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 22
(D) OTHER INFORMATION: /product= "OTHER"
/note= "The carboxy terminus is amidated."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Arg Asp Cys Cys Thr Xaa Xaa Lys Lys Cys Lys Asp Arg Arg Cys Lys
1 5 10 15

Xaa Leu Lys Cys Cys Ala
20

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 84 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Conus purpurascens

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..81

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```
GAA AAG AGA CAA CGA CTG TGT TGC GGT TTT CCG AAG AGT TGC AGA TCC          48
Glu Lys Arg Gln Arg Leu Cys Cys Gly Phe Pro Lys Ser Cys Arg Ser
 35                  40                  45

CGA CAA TGC AAA CCT CAT AGG TGT TGC GGA GGA TAA                       84
Arg Gln Cys Lys Pro His Arg Cys Cys Gly Gly
 50                  55
```

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```
Glu Lys Arg Gln Arg Leu Cys Cys Gly Phe Prc Lys Ser Cys Arg Ser
 1               5                  10                  15

Arg Gln Cys Lys Pro His Arg Cys Cys Gly Gly
            20                  25
```

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 73 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```
Met Lys Leu Thr Cys Val Val Ile Val Ala Val Leu Leu Leu Thr Ala
 1               5                  10                  15

Cys Gln Leu Ile Thr Ala Asp Asp Ser Arg Gly Thr Gln Lys His Arg
            20                  25                  30
```

Ala Leu Gly Ser Thr Thr Glu Leu Ser Leu Ser Thr Arg Cys Lys Ser
35 40 45

Pro Gly Ser Ser Cys Ser Pro Thr Ser Tyr Asn Cys Cys Arg Ser Cys
50 55 60

Asn Pro Tyr Thr Lys Arg Cys Tyr Gly
65 70

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 78 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Met Lys Leu Thr Cys Met Met Ile Val Ala Val Leu Phe Leu Thr Ala
1 5 10 15

Trp Thr Phe Ala Thr Ala Asp Asp Pro Arg Asn Gly Leu Gly Asn Leu
20 25 30

Phe Ser Asn Ala His His Glu Met Lys Asn Pro Glu Ala Ser Lys Leu
35 40 45

Asn Lys Arg Trp Cys Lys Gln Ser Gly Glu Met Cys Asn Leu Leu Asp
50 55 60

Gln Asn Cys Cys Asp Gly Tyr Cys Ile Val Leu Val Cys Thr
65 70 75

WHAT IS CLAIMED IS:

1. A substantially pure conotoxin selected from the group consisting of δ-conotoxin PVIA consisting of the amino acid sequence Glu-Ala-Cys-Tyr-Ala-Xaa_1-Gly-Thr-Phe-Cys-Gly-Ile-Lys-Xaa_2-Gly-Leu-Cys-Cys-Ser-Glu-Phe-Cys-Leu-Pro-Gly-Val-Cys-Pro-Gly (SEQ ID NO:1), wherein Xaa_1 or Xaa_2 is Pro or *4-trans*-hydroxyproline, and μ-conotoxin PIIIA consisting of the amino acid sequence Xaa_3-Arg-Leu-Cys-Cys-Gly-Phe-Xaa_4-Lys-Ser-Cys-Arg-Ser-Arg-Gln-Cys-Lys-Xaa_4-His-Arg-Cys-Cys (SEQ ID NO: 2) wherein Xaa_3 represents pyroglutamate or glutamine and Xaa_4 represents 4-trans-hydroxyproline or proline.

2. A conotoxin according to claim 1 wherein said conotoxin is a substantially pure δ-conotoxin PVIA having the formula Glu-Ala-Cys-Tyr-Ala-Xaa_1-Gly-Thr-Phe-Cys-Gly-Ile-Lys-Xaa_2-Gly-Leu-Cys-Cys-Ser-Glu-Phe-Cys-Leu-Pro-Gly-Val-Cys-Pro-Gly (SEQ ID NO:1), wherein Xaa_1 or Xaa_2 is Pro or *4-trans*-hydroxyproline.

3. The peptide of claim 2 wherein Xaa_1 is *4-trans*-hydroxyproline.

4. The peptide of claim 2 wherein Xaa_1 is Pro.

5. The peptide of claim 2 wherein Xaa_2 is *4-trans*-hydroxyproline.

6. The peptide of claim 2 wherein Xaa_2 is Pro.

7. The peptide of claim 3 wherein Xaa_2 is Pro.

8. The peptide of claim 3 wherein Xaa_2 is *4-trans*-hydroxyproline.

9. The peptide of claim 4 wherein Xaa_2 is Pro.

10. The peptide of claim 4 wherein Xaa_2 is *4-trans*-hydroxyproline.

11. A conotoxin according to claim 1 wherein said conotoxin is a substantially pure μ-conotoxin PIIIA peptide consisting of the amino acid sequence Xaa_1-Arg-Leu-Cys-Cys-Gly-Phe-Xaa_2-Lys-Ser-Cys-Arg-Ser-Arg-Gln-Cys-Lys-Xaa_2-His-Arg-Cys-Cys (SEQ ID NO: 2) where Xaa_1 represents pyroglutamate or glutamine and Xaa_2 represents 4-trans-hydroxyproline or proline.

12. The peptide of claim 11 wherein the carboxy terminus is amidated.

13. An isolated nucleic acid consisting essentially of the sequence 5`-GAAAAGAGACAACGACTGTGTTGCGGTTTTCCGAAGAGTTGCAGATCCCGACA ATGCAAACCTCATAGGTGTTGCGGAGGATAA-3` (SEQ ID NO: 11).

14. A substantially pure peptide consisting essentially of the amino acid sequence Glu-Lys-Arg-Gln-Arg-Leu-Cys-Cys-Gly-Phe-Pro-Lys-Ser-Cys-Arg-Ser-Arg-Gln-Cys-Lys-Pro-His-Arg-Cys-Cys-Gly-Arg (SEQ ID NO:12).

signal sequence

1. met lys leu thr cys val val ile val ala val leu leu leu thr ala
2. met lys leu thr cys met met ile val ala val leu phe leu thr ala
3. met lys leu thr cys val met ile val ala val leu phe leu thr ala

ATG AAA CTG ACG TGC GTG ATG ATC GTT GCT GTG CTG TTC TTG ACT GCC

cys gln leu ile thr ala asp asp ser arg --- gly thr gln lys his
trp thr phe ala thr ala asp asp pro arg asn gly leu gly asn leu
trp thr phe val thr ala asp asp ser lys asn gly leu glu asn his

TGG ACA TTC GTC ACG GCT GAT GAC TCC AAA AAT GGA CTG GAG AAT CAT

--- --- arg ala leu gly ser thr thr --- --- glu leu ser --- leu
phe ser asn ala his his glu met lys asn pro glu ala ser lys leu
phe trp lys ala arg asp glu met lys asn arg glu ala ser lys leu

TTT TGG AAG GCA CGT GAC GAA ATG AAG AAC CGC GAA GCC TCT AAA TTG

ser thr arg --- --- cys lys ser pro gly ser ser cys ser pro thr
asn lys arg --- trp cys lys gln ser gly glu met cys asn leu leu
asp lys lys glu ala cys tyr ala pro gly thr phe cys gly ile lys

GAC AAA AAG GAA GCC TGC TAT GCG CCT GGT ACT TTT TGT GGC ATA AAG

mature toxin

ser tyr asn cys cys --- arg ser cys asn pro tyr thr lys arg cys
asp gln asn cys cys asp gly tyr cys ile val leu val --- --- cys
pro gly leu cys cys ser glu phe cys leu pro gly val --- --- cys

CCC GGG CTA TGC TGC AGT GAG TTT TGT CTC CCG GGC GTC --- --- TGC

tyr gly	SEQ ID NO:13
thr	SEQ ID NO:14
phe gly gly	SEQ ID NO:5
TTC GGT GGT	SEQ ID NO:4

FIG. 1

SUBSTITUTE SHEET (RULE 26)

--- GAA AAG AGA CAA CGA CTG TGT TGC GGT TTT CCG AAG AGT TGC AGA
Glu Lys Arg Gln Arg Leu Cys Cys Gly Phe Pro Lys Ser Cys Arg

TCC CGA CAA TGC AAA CCT CAT AGG TGT TGC GGA CGA TAA --- SEQ ID NO:11
Ser Arg Gln Cys Lys Pro His Arg Cys Cys Gly Arg OCH SEQ ID NO:12

FIG. 2A

(1) (2) (3) (4)

---EKRQRLCCGFPKSCRSRQCKPHRCCGR SEQ ID NO: 12

⇓

ZRLCCGFOKSCRSRQCKOHRCC-NH_2 SEQ ID NO: 2

FIG. 2B

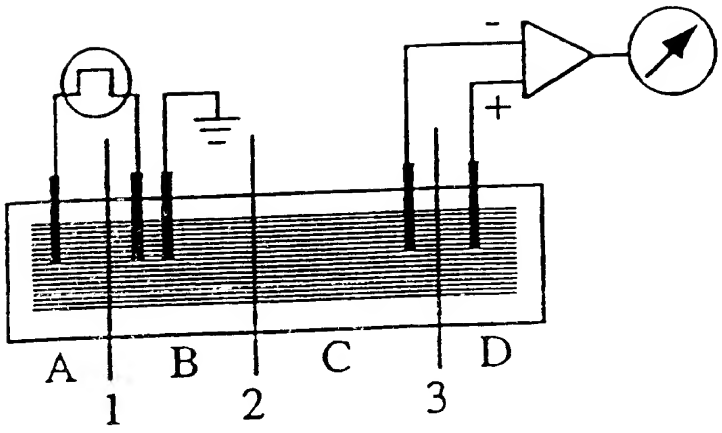


FIG. 3A

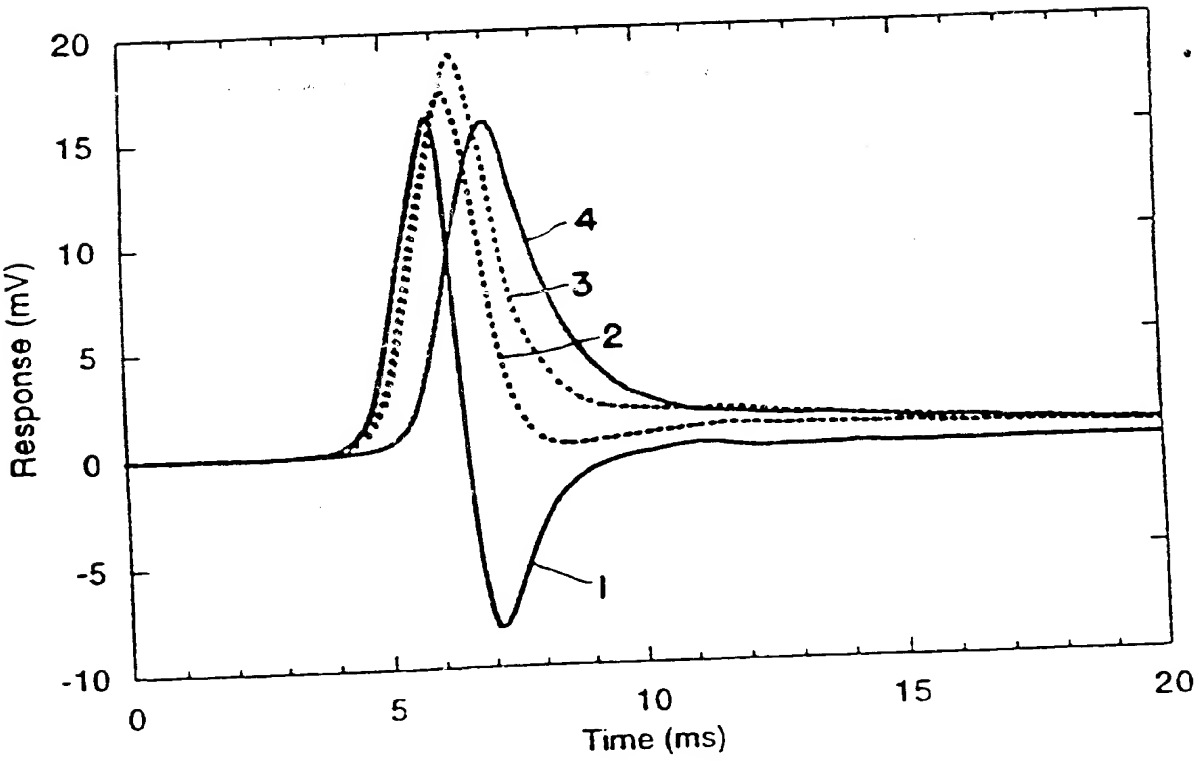


FIG. 3B

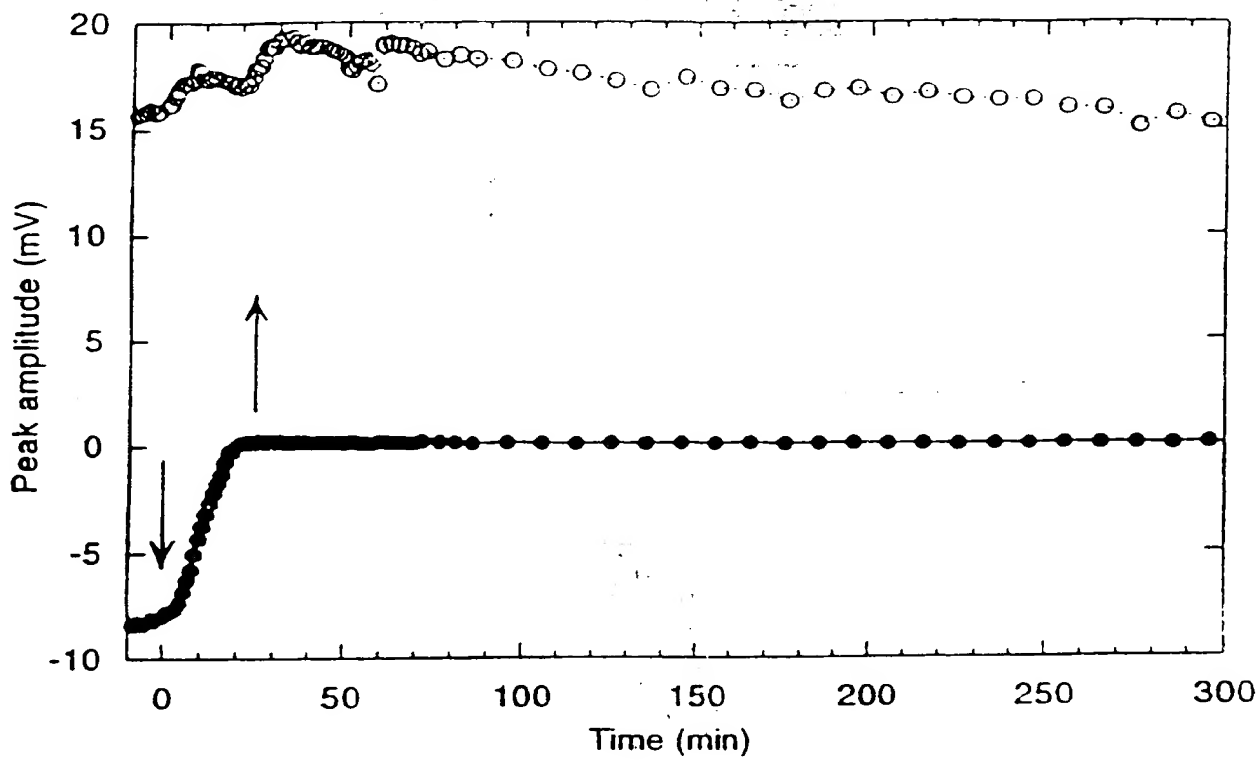


FIG. 3C

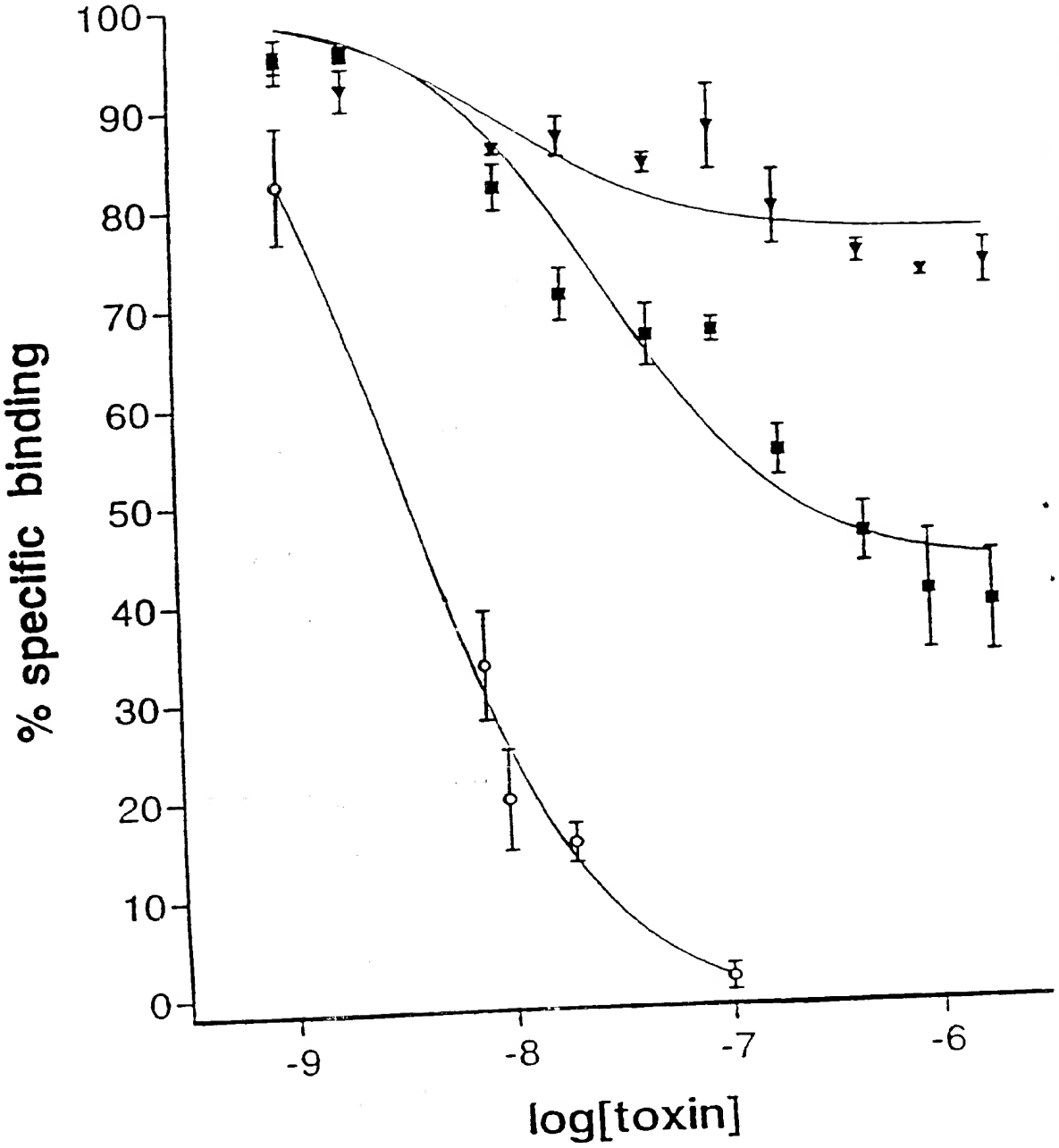


FIG. 4

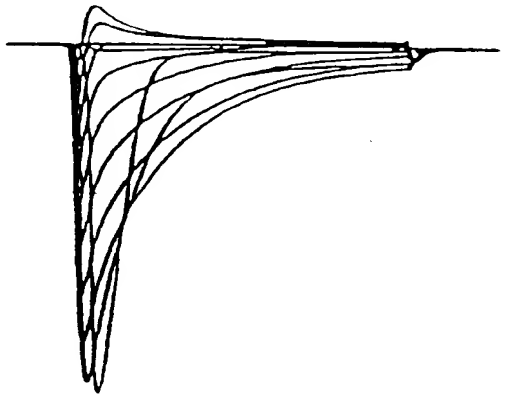


FIG. 5A

FIG. 5B

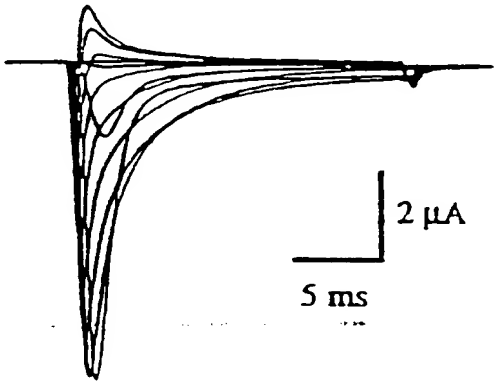


FIG. 5C

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/05262

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07H 21/04; C07K 14/435
US CL :514/12, 13; 530/324, 326; 536/23.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/12, 13; 530/324, 326; 536/23.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG, STN, SWISS-PROT, GENESEQ, PIR
search terms: conotoxin, conus, sodium channel, PVIA, PIIIA, SEQ ID NOS:1, 2, 12

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,P	US 5,432,155 A (OLIVERA ET AL) 11 July 1995 .	1-14
A	FAINZILBER et al. A New Conotoxin Affecting Sodium Current Inactivation Interacts with the δ-Conotoxin Receptor Site. The Journal Of Biological Chemistry. 20 January 1995, Volume 270, Number 3, pages 1123-1129.	1-14
A	MYERS et al. Conus Peptides as Chemical Probes for Receptors and Ion Channels. Chemical Reviews. 1993, Volume 93, Number 5, pages 1923-1936.	1-14
A	SHON et al. δ-Conotoxin GmVIA, a Novel Peptide from the Venom of Conus gloriamaris. Biochemistry. 1994, Volume 33, Number 38, pages 11420-11425.	1-14

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
27 JUNE 1996

Date of mailing of the international search report
01 AUG 1996

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231
Facsimile No. (703) 305-3230

Authorized officer
JEFFREY E. RUSSEL

Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/05262

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	SHON et al. Purification, Characterization, Synthesis, and Cloning of the Lockjaw Peptide from Conus purpurascens Venom. Biochemistry. 18 April 1995, Volume 34, Number 15, pages 4913-4918, especially page 4914, column 1, first and second full paragraphs, page 4915, column 2, line 12.	1-3, 5, 8